DESIGN, PRODUCTION AND CHARACTERISATION OF IGF-I ANALOGUES WITH INCREASED GASTRIC STABILITY

by

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ABSTRACT

Although insulin-like growth factor I (IGF-I), and its analogue long-R^3-IGF-I, are potent gut growth factors, their potential bio-activity when administered orally is limited by rapid degradation in the stomach and other regions of the gut. In this study, I have introduced single-site mutations into long-R^3-IGF-I to produce analogues with enhanced stability against purified porcine pepsin and retained bio-activity.

Five initial cleavage sites of porcine pepsin digestion were determined for the *N*-terminally extended IGF-I variant, long-R^3-IGF-I. Putatively pepsin-resistant analogues were constructed using site directed mutagenesis at the most susceptible sites. The single point mutations made were Leu10Val, Phe16Ala, Phe25Leu, Asp53Glu and Met59Gln in the IGF-I moiety.

Biological activities of the analogues were assessed using assays measuring stimulation of protein synthesis using rat L6 myoblasts, type 1 IGF-receptor affinity as determined in rat L6 myoblasts and affinity for the IGF binding proteins as secreted by L6 myoblasts into conditioned media. The mutations Leu10Val and Phe25Leu had reduced type 1 receptor binding, whereas Phe16Ala, Asp53Glu and Met59Gln had limited effect. Relative binding of the mutants to IGF binding proteins showed Asp53Glu had a slightly increased affinity compared to long-R^3-IGF-I. Phe25Leu and Met59Gln mutants had affinities similar to that of the parent molecule, while Leu10Val and Phe16Ala both showed marked decreases in IGF binding protein affinity.

Stabilities of the analogues to purified porcine pepsin were assessed using reverse-phase high performance liquid chromatography. Pepsin digestion of the mutants showed the Phe16Ala mutation had the greatest pepsin stability, being stabilised approximately 10-fold compared to long-R^3-IGF-I. The mutations, Phe25Leu and Met59Gln, had a 4 and 3-fold higher stability, respectively, while Leu10Val and Asp53Glu were equivalent or slightly less pepsin-resistant than long-R^3-IGF-I.

The relative stability of these peptides was then evaluated in rat stomach flushings using human placental membrane assays. The analogue containing the Phe16Ala mutation, long-R^3A^{16}-IGF-I, showed a 2-fold higher stability than long-R^3-IGF-I when determined using unlabelled or iodinated peptides. Addition of an aspartic protease inhibitor, aprotinin, to the flushings almost completely abolished peptide degradation. Other single-site mutations, Leu10Val, Phe25Leu, Asp53Glu and Met59Gln, yielded stabilities intermediate between those of long-R^3-IGF-I and long-R^3A^{16}-IGF-I, when digested with rat stomach flushings. Comparisons using human and pig stomach flushings showed that although the flushings varied in their ability to degrade the long-R^3-IGF-I analogues, long-R^3A^{16}-IGF-I was stabilised approximately 2-fold compared to the parent material, long-R^3-IGF-I, in all cases.

As long-R^3A^{16}-IGF-I showed the highest pepsin stability of the analogues produced, the relative stability of long-R^3A^{16}-IGF-I and long-R^3-IGF-I was then evaluated under a range of conditions that reflect the variable stomach environment *in vivo* using placental membrane binding assays. The Phe16Ala mutation stabilised long-R^3-IGF-I against porcine pepsin A up to 8-fold in 10 mM HCl, pH 2.0, although the stability advantage was diminished at higher pepsin : substrate ratios. Both long-R^3-IGF-I and long-R^3A^{16}-IGF-I were more resistant to pepsin when incubated in 10 mM HCl, pH 2.0, than in 50 mM glycine, pH 3.2. The addition of 154 mM NaCl to both buffer systems decreased the stabilities of the peptides.

This study has shown that single point mutations can be introduced in long-R^3-IGF-I to increase the stability towards pepsin digestion while still retaining biological activity. Of the mutants produced, the Phe16Ala mutation was the most effective. Although the stability advantage of long-R^3A^{16}-IGF-I is dependent on pH, salt concentrations or enzyme : substrate ratio, it is consistently more stable than the parent long-R^3-IGF-I against purified pepsin or stomach flushings from several species.

STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief it contains no material that has previously been published by any other person except where due reference is made. The author consents to the thesis being made available for photocopying and loan.

Katherine Bryant

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ABBREVIATIONS

The following abbreviations were used in addition to those abbreviations commonly accepted.

2-amino-2-hydroxymethylpropane-1,3-diol	Tris
4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid	Hepes
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	BCIG
acid labile subunit	ALS
adrenocorticotropic hormone	ACTH
basic fibroblast growth factor	bFGF
bovine serum albumin	BSA
calf intestinal alkaline phosphatase	CIP
cerebral spinal fluid	CSF
Co-operative research centre	CRC
counts per minute	c.p.m.
dithioerythritol	DTE
dithiothreitol	DTT
Dulbecco's Modified Eagle's Medium	DMEM
Escherichia coli	*E. coli*
ethylenediaminetetraacetic acid	EDTA
fast protein liquid chromatography	FPLC
fibroblast growth factor	FGF
follicle stimulating hormone	FSH
growth hormone	GH
high performance liquid chromatography	HPLC
insulin-like growth factor	IGF : IGF-I and IGF-II
insulin-like growth factor binding protein	IGFBP : IGFBP-1 to IGFBP-6
isopropyl-β-D-thiogalactopyranoside	IPTG

Luria-Bertani	LB
[Met^{1}]-pGH(1-11)-Val-Asn-[Arg^{3}]-IGF-I	long-R^{3}-IGF-I
[Met^{1}]-pGH(1-11)-Val-Asn-[Arg^{3}, Ala^{16}]-IGF-I	long-R^{3}A^{16}-IGF-I
[Met^{1}]-pGH(1-11)-Val-Asn-[Arg^{3}, Glu^{53}]-IGF-I	long-R^{3}E^{53}-IGF-I
[Met^{1}]-pGH(1-11)-Val-Asn-[Arg^{3}, Gln^{59}]-IGF-I	long-R^{3}Q^{59}-IGF-I
[Met^{1}]-pGH(1-11)-Val-Asn-[Arg^{3}, Leu^{25}]-IGF-I	long-R^{3}L^{25}-IGF-I
[Met^{1}]-pGH(1-11)-Val-Asn-[Arg^{3}, Val^{10}]-IGF-I	long-R^{3}V^{10}-IGF-I
nuclear magnetic resonance	NMR
Phe-Val-Asn-[Arg^{3}]-IGF-I	FVN-R^{3}-IGF-I
Phe-Val-Asn-[Arg^{3}, Ala^{16}]-IGF-I	FVN-R^{3}A^{16}-IGF-I
phenylmethylsulfonyl fluoride	PMSF
platelet-derived growth factor	PDFG
porcine growth hormone	pGH
sodium dodecyl sulphate	SDS
standard error of the mean	SEM
trichloroacetic acid	TCA
trifluoroacetic acid	TFA
ultra violet	UV

PUBLICATIONS ARISING FROM THIS THESIS

Bryant, K. J., Forsberg, G., Read, L. C. & Wallace, J. C. (1995) Design and characterization of long-R^3-insulin-like growth factor I mutants which show resistance to pepsin digestion. *Biochemical Journal* (submitted).

Bryant, K. J., Forsberg, G., Xian, C. J., Wallace, J. C. & Read, L. C. (1995) Degradation of long-R^3-insulin-like growth factor I analogues by purified porcine pepsin and stomach flushings from pig, rat and human. *Journal of Endocrinology* (submitted).

CHAPTER ONE

THE INSULIN-LIKE GROWTH FACTOR PEPTIDES: THEIR PRESENCE AND ACTIONS IN THE GASTROINTESTINAL TRACT AND STABILITY TO THE GASTRIC PROTEINASE, PEPSIN.

The main objective of this thesis is to examine whether insulin-like growth factor I (IGF-I) could be stabilised against proteolytic degradation thereby allowing oral applications to the gastrointestinal tract.

This review examines (1) characteristics of the IGFs with respect to their structure, distribution, action and regulation, (2) the IGF-receptors and IGF-binding proteins, (3) IGF structure-function relationships, (4) the presence, actions and survival of the IGFs in the gastrointestinal tract, then finally, (5) the stomach environment and the gastric proteinases.

In order to determine the logic leading to initiation of the project, this review generally cites work published prior to April 1992, the commencement of my PhD research, except for reference to general review articles. Information published since mid-1992 which is relevant to this thesis is included in the appropriate discussion sections of each chapter.

1.1 DEFINITION OF GROWTH FACTORS

Peptides with a regulatory action on tissue growth, either stimulatory or inhibitory, have been termed growth factors. Many of these growth factors were subsequently named according to their biological activities, such as epidermal growth factor and fibroblast growth factor (FGF). It is now evident that growth factors are a diverse group, many of which are multi-functional agents, often having a range of biological activities including effects unrelated to the control of cell growth (James & Bradshaw, 1984; Sporn & Roberts, 1988; Herndon *et al.*, 1992).

In contrast to hormones, which are produced by specialised endocrine glands, growth factors are synthesised and secreted by a wide variety of cells and tissue types. Most growth factors are not stored in vesicles in their synthesising cells. On secretion, they diffuse through the extracellular fluids to their sites of action, which can include endocrine, paracrine or autocrine mechanisms (James & Bradshaw, 1984).

All growth factors interact with a specific, high affinity, cell surface receptor to initiate a response. This is followed by internalisation of the ligand and receptor by receptor-mediated endocytosis, then degradation with possible receptor recycling. The specific responses for all the growth factors are distinct, mediated by different mechanisms of action. Binding of the growth factors to their specific receptors results in a cascade of events which generally produces a trophic stimulation that is characterised by metabolic uptake, modulation of ion fluxes and an increase in the anabolic metabolism of the cell. With many growth factor receptors, ligand binding induces activation of an associated tyrosine kinase. This activation results in phosphorylation of various intracellular proteins as well as autophosphorylation of the receptor, an event which can act as a regulatory function for the kinase activity. Receptor binding may also modulate expression of specific genes either at the transcriptional and/or translational levels, with protein synthesis generally being stimulated (James & Bradshaw, 1984; Yarden & Ullrich, 1988).

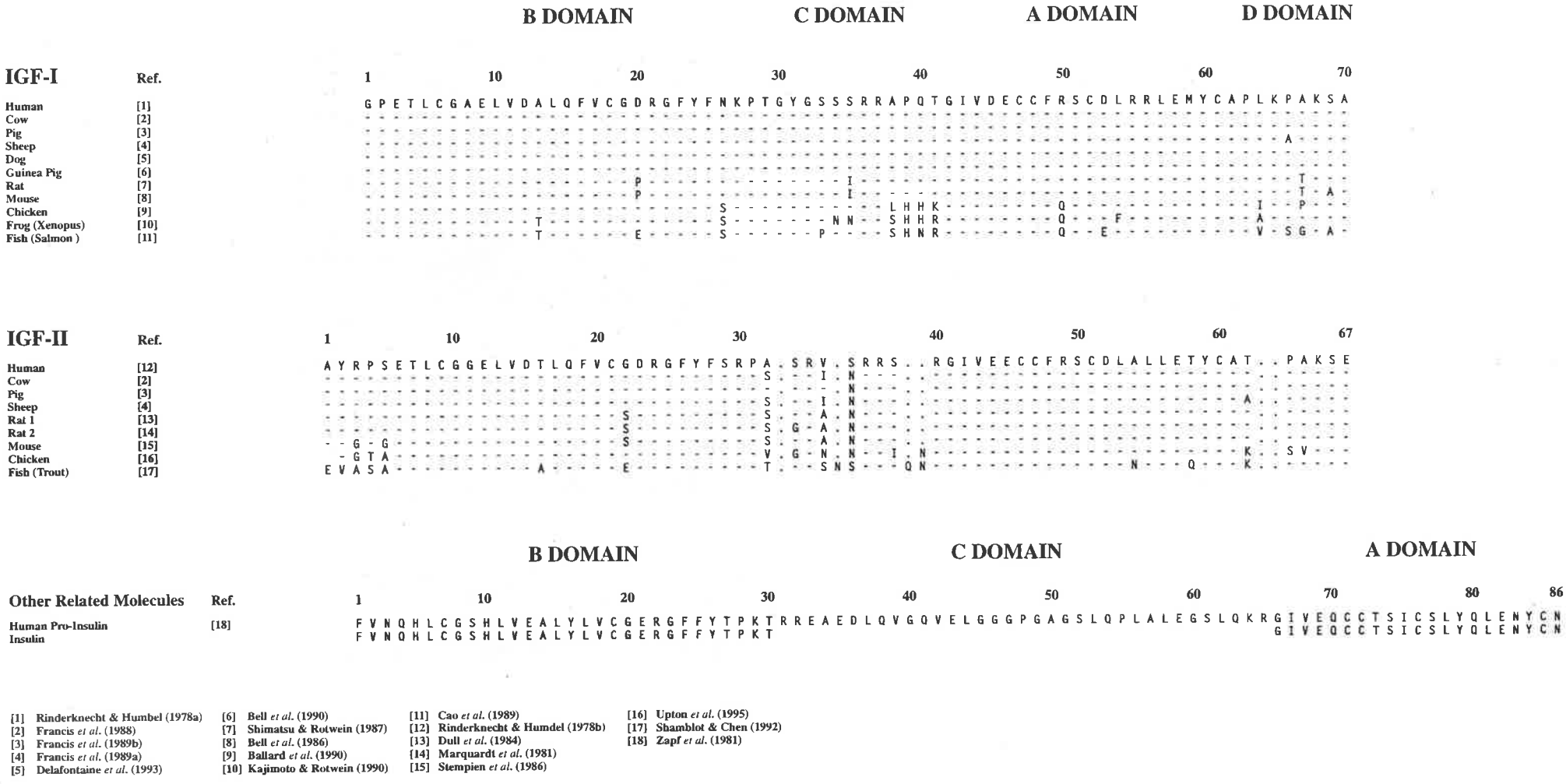
1.2 THE INSULIN-LIKE GROWTH FACTORS

1.2.1 Historical identification of the insulin-like growth factors

The insulin-like growth factors (IGFs) were first discovered as three separate biological activities in serum. In 1957, Salmon and Daughaday reported that serum from normal rats, but not from hypophysectomized rats, could stimulate uptake of sulphate into cartilage, and designated the factor, 'sulfation factor activity'. Froesch *et al.* (1963) found serum had insulin-like effects on adipose tissue in the presence of insulin antibodies. This was consequently termed 'non-suppressible insulin-like activity'. Then, a 'multiplication-stimulation activity' for chick embryo fibroblasts was purified from calf serum by Pierson & Temin (1972). When it became apparent that these three activities represented a similar group of substances with a wider range of biological activities, the term 'somatomedin' was introduced to denote factors that mediate the action of growth hormone in stimulating somatic growth as well as displaying insulin-like activity (Daughaday *et al.*, 1972). Further identification at both the gene and protein level showed biological activities in human serum was attributed to two different peptides, 'insulin-like growth factor I and II' (IGF-I and IGF-II) and their variant forms (Rinderknecht & Humbel, 1976, 1978a & 1978b; Klapper *et al.*, 1983; Enberg *et al.*, 1984). Since IGF-II was found to be less growth hormone-dependent than IGF-I/somatomedin-C (Rinderknecht & Humbel, 1978b; Baxter, 1986), it was recommended that the term IGF be used to denote this family of peptides (Daughaday *et al.*, 1987).

1.2.2 IGF protein structure

IGF-I and IGF-II are single chain polypeptides consisting of 70 and 67 amino acids respectively. These two peptides were first characterised from human plasma by Rinderknecht & Humbel (1978a & 1978b) and subsequently have now been purified from many species. The sequence homology is highly conserved between mammals, with human, bovine and porcine IGF-I having an identical sequence, while ovine and rat IGF-I have one and three amino acid differences respectively. A similarly conserved amino acid sequence is also shown between mammalian IGF-IIs (Figure 1.1).



[1] Rinderknecht & Humbel (1978a)
[2] Francis *et al.* (1988)
[3] Francis *et al.* (1989b)
[4] Francis *et al.* (1989a)
[5] Delafontaine *et al.* (1993)
[6] Bell *et al.* (1990)
[7] Shimatsu & Rotwein (1987)
[8] Bell *et al.* (1986)
[9] Ballard *et al.* (1990)
[10] Kajimoto & Rotwein (1990)
[11] Cao *et al.* (1989)
[12] Rinderknecht & Humdel (1978b)
[13] Dull *et al.* (1984)
[14] Marquardt *et al.* (1981)
[15] Stempien *et al.* (1986)
[16] Upton *et al.* (1995)
[17] Shamblot & Chen (1992)
[18] Zapf *et al.* (1981)

FIGURE 1.1
Comparative sequence data of the IGF growth factors.
Dashed lines represent identical sequence compared to respective human counterparts. Periods were used to frame shift peptides for maximal alignment between sequences. Complete sequences are given for other related molecules. (Adapted from a leaflet produced by GroPep Ltd., Adelaide, SA, Australia)

IGF-I and IGF-II show strong homology to each other and with other members of the insulin family, which include the insulins, the relaxins, the bombyxins and the molluscan insulin-like peptides (Murray-Rust *et al.*, 1992; Blundell & Humbel, 1980). Figure 1.2 is a schematic representation of the predicted folded structures for various members of the insulin family. In the human, IGF-I shows 62 % and 49 % sequence homology respectively to IGF-II and insulin. In addition, the six cysteine residues are conserved forming the same three disulphide bonds. These molecules also show structural homology to each other and can be described in domains analogous to the *A*, *B* and *C* chains of proinsulin (Humbel, 1990). The *C*-domains of IGF-I and IGF-II contain 12 amino acids. This domain is analogous to the 35 amino acid *C*-domain cleaved from proinsulin to form insulin. In addition and unlike proinsulin, the IGFs also contain a carboxyl terminal extension which has been labelled the *D*-region. Figure 1.1 shows comparative sequence data for the IGFs and insulin.

Using X-ray crystallography and solution structure ^{1}H-NMR spectroscopy, three-dimensional models for IGF-I and IGF-II have been constructed (Blundell *et al.*, 1978; Cooke, *et al.*, 1991; Sato, *et al.*, 1992 & 1993; Torres *et al.*, 1995). As with insulin, the IGFs have a core structure consisting of three α-helices in the *A* and *B*-domains. The *C*-domain, connecting the helices, is an extended β-turn conformation. Both the *C*- and *D*-domains are poorly defined, indicating flexibility in these regions of the IGF molecule.

1.2.3 IGF gene structure

The IGF-I and IGF-II genes from human and rat are the most extensively characterised, showing complex organisational and expression patterns (Sussenbach, 1989). The IGF genes are controlled by multiple promoters, which regulate the gene expression in a tissue-specific and developmental-dependent manner. The human IGF-I gene, which has been mapped on the long arm of chromosome 12, is estimated to be approximately 90 kb of chromosomal DNA with six exons and at least two promoters (Jansen *et al.*, 1992). In both human and rat multiple mRNA species, ranging in length from 0.8 to 7.6 kb, are generated by alternate splicing of the primary transcript and encode at least two different types of precursor proteins. In several non-hepatic tissues of the rat, including the gut, a higher ratio

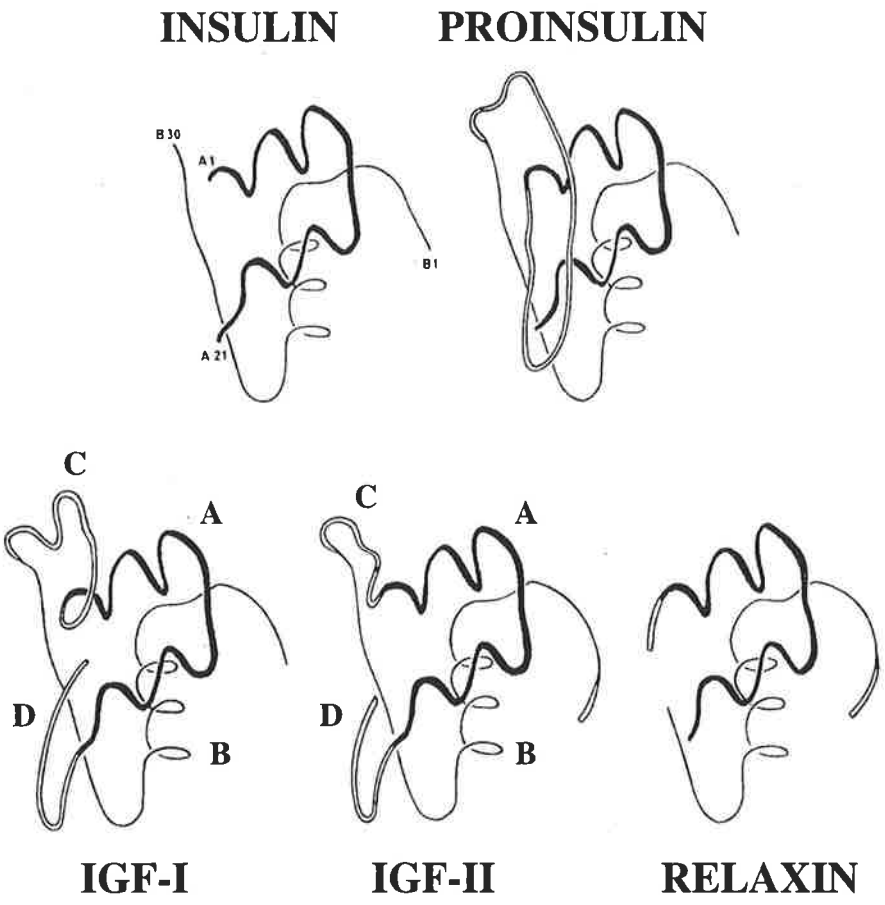


FIGURE 1.2
Schematic structure of the insulin family.
Predicted folding structures for IGF-I, IGF-II, insulin, proinsulin and relaxin are shown, indicating the structural similarity of the insulin family. The *A*-domain is indicated in black, the *B*-domain by a single line, the *C*-region by the double line between the *A*- and *B*-domains and the *D*-region by the double line extending from the *A*-domain at the C-terminus. Insulin lacks the *C*- and *D*-regions. (Adapted from Blundell and Humbel, 1980)

of large : small molecular weight IGF-I mRNAs are expressed than in the liver, which suggests that in these tissues, post-transcriptional regulation of IGF-I synthesis may be of particular importance (Lund *et al.*, 1986). The human IGF-II gene is equally as complex, containing at least four promoters and 9 exons, spanning a region of about 30 kb of chromosomal DNA on the short arm of chromosome 11 (Van Dijk *et al.*, 1991).

Both IGF-I and IGF-II are produced as pre-pro-peptides with a signal sequence and a carboxyl terminal *E*-peptide (Rotwein, 1991; Daughaday & Rotwein, 1989; Lund, 1994). The mature IGFs are obtained by post-translational proteolytic processing (Rotwein *et al.*, 1987a; Bach *et al.*, 1990).

1.2.4 Distribution and localisation of IGFs

The IGFs are present in various body fluids, including serum (Zapf *et al*, 1981), urine (Hizuka *et al.*, 1987), cerebral spinal fluid (CSF) (Haselbacher and Humbel, 1982), seminal fluid (Baxter *et al.*, 1984a), saliva (Costigan *et al.*, 1988), and mammary secretions (Baxter *et al.*, 1984b). The IGFs are also synthesised in many cell and tissue types, both in the fetus and in the adult (D'Ercole *et al.*, 1984 & 1986). Tissue IGF levels however, generally tend to be much lower compared with serum or plasma (D'Ercole *et al.*, 1984). The liver has been implicated as the primary source of the circulating IGFs in serum (D'Ercole *et al.*, 1984; Froesch *et al.*, 1985; Baxter, 1986; Lund *et al.*, 1986).

1.2.5 IGF actions

As with other growth factors, the biological actions of IGFs are diverse and are not limited only to growth. It is now apparent that the IGFs have roles involving paracrine and autocrine modes of action, as well as acting as classic endocrine hormones (Holly & Wass 1989; Daughaday & Rotwein, 1989; LeRoith & Roberts, 1991).

The *in vitro* action of the IGFs have been well established in a variety of cell types (Sara & Hall, 1990; Lowe, 1991; Jones & Clemmons, 1995). In general, the effects of the IGFs *in vitro* are either acute anabolic effects on protein and carbohydrate metabolism, or longer term effects on cell replication and differentiation. Anabolic effects include stimulation

of glucose uptake and metabolism, increased amino acid uptake as well as stimulation of RNA and protein synthesis (Lowe, 1991). These effects occur in a variety of cell types, including myoblasts, fibroblasts, chondrocytes and epithelial cells in the gut (Ballard *et al.*, 1986; Hill *et al.*, 1986; Kemp *et al.*, 1988; Olanrewaju *et al.*, 1992). For the more long term growth-promoting effects, such as on DNA synthesis and cell proliferation, the IGFs must interact with other exogenous or endogenous growth factors, such as platelet-derived growth factor (PDGF) and FGF (Lowe, 1991). It has been suggested the IGFs are acting as progression factors in conjunction with other growth factors (PDGF and FGF) which are acting as competence factors (Stiles *et al.*, 1979; Leof *et al.*, 1982). Apart from stimulating growth, the IGFs can also promote cellular differentiation (Schmid *et al.*, 1983 & 1984; McMorris *et al.*, 1986; Smith *et al.*, 1988) and have effects on cell function, such as stimulating progesterone and oestrogen synthesis and secretion in ovarian granulosa cells (Adashi *et al.*, 1984; Veldhuis *et al.*, 1986).

More recently, the availability of IGFs synthesised by recombinant DNA technology has provided the means for assessing the metabolic actions of the IGFs *in vivo*. The biological activity of the IGFs has been demonstrated in a number of *in vivo* models, including the neonate (Philipps *et al.*, 1988), hypophysectomized (Skottner *et al.*, 1987), diabetic (Scheiwiller *et al.*, 1986) and normal (Hizuka *et al.*, 1986) rats, using either continuous infusions or intravenous boluses. In general, the anabolic and growth promoting effects of the IGFs which are shown *in vitro* can also be demonstrated *in vivo*. For example, the acute metabolic actions of the IGFs were first reported by Zapf *et al.* (1986) who showed that intravenous injections of either IGF-I or IGF-II induced hypoglycaemic effects in normal rats by enhancing glucose uptake from the serum. Anabolic effects associated with protein synthesis and nitrogen balance have also been reported in several animal and human studies (Pell & Bates, 1992; Tomas *et al.*, 1992; Skottner *et al.*, 1987; Guler *et al.*, 1987). Similarly, the more long term growth promoting actions of the IGFs have also been shown (Skottner *et al.*, 1987 & 1989; Schoenle *et al.*, 1985; Guler *et al.*, 1988).

Transgenic animal models have also been useful in establishing the effects of IGFs *in vivo*. These experiments suggest that a large majority of the effects of GH are mediated by

IGF-I, and that variations in local expression of IGF-I can result in disproportionate growth of specific tissues (Mathews *et al.*, 1988; Behringer *et al.*, 1990). Mice with null mutations for genes encoding IGF-I, IGF-II and the IGF-receptors, have demonstrated the importance of IGF-I and IGF-II during fetal development (reviewed by Jones & Clemmons, 1995).

1.2.6 Regulation of IGF expression

As to be expected with a growth factor exhibiting such variable actions, IGF expression is regulated in multiple ways (Daughaday & Rotwein, 1989; Sara & Hall, 1990; Adamo *et al.*, 1991).

Tissue specific factors play an important role in modulating the IGFs gene expression (Soares *et al.*, 1985; Brown *et al.*, 1986a; Murphy *et al.*, 1987; Han *et al.*, 1988). For example, in the adult rat, IGF-I mRNA is present in many tissues although the abundance varies, with liver having the highest concentration (Lund *et al.*, 1986; Murphy *et al.*, 1987). Within tissues, the cellular sites of IGF gene expression can vary by both cell type and region (Hernandez *et al.*, 1989 & 1990). IGF-I and IGF-II are also developmentally regulated. IGF-I, for example, is expressed in greater levels in rat adult liver compared to fetal liver (Rotwein, 1986; Rotwein *et al.*, 1987b) while IGF-II, which is present in many fetal and embryonic structures, becomes restricted to specific tissues such as the liver, kidney and brain in the late gestation human fetus or in the adult rat (Scott *et al.*, 1985; Han *et al.*, 1987b; Lund *et al.*, 1986).

In most tissues growth hormone (GH) is the major trophic factor regulating IGF-I post-natally. The action of GH is rapid and causes an increase in IGF-I mRNA abundance (Mathews *et al.*, 1986; Lowe *et al*, 1987 & 1988). IGF-II mRNA levels, however, do not show the same strong GH dependency as IGF-I. This is shown, for example, in adult rats where hypophysectomy reduces hepatic levels of IGF-I mRNA (D'Ercole *et al.*, 1984) but does not further reduce the level of IGF-II mRNA (Hynes *et al.*, 1987).

IGF-I and IGF-II protein and mRNAs are also regulated by many other hormones and trophic factors. PDGF, FGF, FSH, lutenizing hormone and adrenocorticotropic hormone (ACTH) are some of the factors which stimulate synthesis of IGF-I, while glucocorticoids

are inhibitory (Sara & Hall, 1990; Rotwein, 1991; Holly & Wass, 1989). In contrast to IGF-I, the trophic factors involved in the regulation of IGF-II expression are not well-defined, however oestrogen, gonadotrophins, glucocorticoids, ACTH and insulin have all been shown to affect IGF-II gene expression (Adamo *et al.*, 1991).

Other modulators in the regulation of IGF production include nutritional status (Emler & Schalch, 1987; Thissen *et al.*, 1994), response to local tissue injury (Sara & Hall, 1990; Adamo *et al.*, 1991) and the production of multiple mRNA transcripts for both IGF-I and IGF-II (Sara & Hall, 1990; Adamo *et al.*, 1991; Ward & Ellis, 1992; Lund., 1994).

1.2.7 IGF-receptors

There are two known receptors which specifically recognise the IGFs: the type 1 and type 2 IGF-receptors (Rechler & Nissley, 1985; Nissley & Lopaczynski, 1991; Jones & Clemmons, 1995). In addition, the IGFs can also bind to the insulin receptor, although with low affinity, and to hybrid IGF/insulin receptors the latter of which have been isolated from solubilised placental membranes (Soos & Siddle, 1989), cultured cells (Moxham *et al.*, 1989) and also assembled *in vitro* (Treadway *et al.*, 1991). Figure 1.3 is a schematic representation of the IGF-receptors and Table 1.1 is a summary of the relative affinities of the IGFs for the receptors.

Most of the mitogenic and metabolic actions of IGFs are mediated through specific interactions with the type 1 receptor (Werner *et al.*, 1991). This receptor has a higher affinity for IGF-I than for IGF-II, and has a further decreased affinity for insulin (Rechler & Nissley, 1985). The type 1 receptor and the insulin receptor are homologous heterotetrameric structures, comprising of two α (135 kDa) and two β (95 kDa) subunits connected by disulphide bonds (Kasuga *et al.*, 1981; Massague & Czech, 1982). The α subunits are located on the outer surface of the plasma membrane and contain the ligand-binding domain while the β subunits span the plasma membrane. The cytoplasmic portion of the β subunits contain a highly conserved tyrosine kinase domain. Ligand binding causes a signalling cascade, resulting in autophosphorylation of the receptor as well as

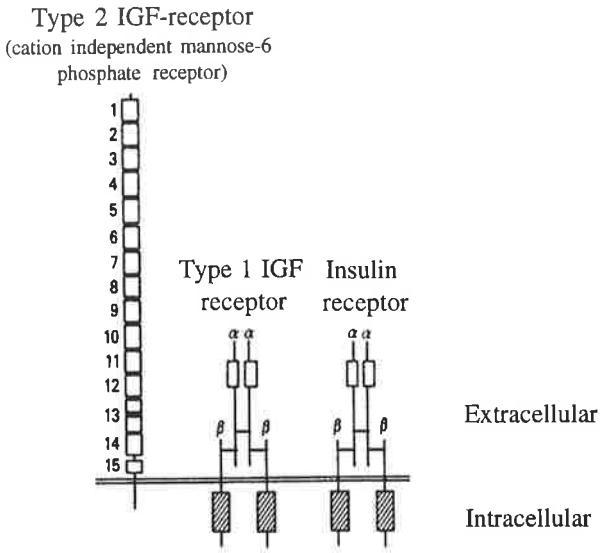


FIGURE 1.3
The insulin-like growth factor receptors.
Schematic representations of the structures of the type 1 and type 2 IGF- and insulin-receptors. Boxed regions in the type 2 IGF-receptor indicate repeat sequences. Boxed regions in the type 1 IGF- and insulin-receptors indicate cysteine rich regions, while hatched boxes indicate the intracellular tyrosine kinase domains. Disulphide bonds joining the subunits within the type 1 IGF- and insulin-receptors are shown. (Adapted from Nissley & Lopacynski, 1991)

RECEPTOR	LIGANDS
Type 1 IGF-Receptor	IGF-I (2-10 X) > IGF-II (100 X) > Insulin
Type 2 IGF-Receptor	IGF-II (1000 X) >> IGF-I [a]
Insulin-Receptor	Insulin (10 X) > IGF-I (100 X) > IGF-II
Hybrid IGF/Insulin-Receptor	IGF-I > Insulin

TABLE 1.1
Relative affinities of the IGFs for the IGF-receptor.
[a] Does not bind insulin. (Adapted from Singh & Rubin, 1993)

phosphorylation of various protein substrates (Jacobs *et al.*, 1985; Izumi *et al.*, 1987; Jones & Clemmons, 1995).

The type 2 IGF-receptor is identical to the cation-independent mannose-6-phosphate receptor, which binds to mannose-6-phosphate residues on lysosomal enzymes (Nissley *et al.*, 1991; Kornfeld, 1992). This is a monomeric receptor of 275 kDa which is present as both a membrane-bound and a soluble form, and is highly specific for IGF-II (Morgan *et al.*, 1987; Kiess *et al.*, 1987; MacDonald, *et al.*, 1989). It is unclear whether this receptor mediates the biological actions of IGF-II, although IGF-II binding causes internalisation and degradation of the growth factor (Oka *et al.*, 1985). Signalling by this receptor may involve GTP binding protein activation (Nishimoto *et al.*, 1989; Murayama *et al.*, 1990). However, a more recent study suggests the type 2 receptor does not function in transmembrane signalling in response to IGF-II (Korner *et al.*, 1995).

1.2.8 IGF binding proteins

In the circulation and throughout the extracellular space, the IGFs are present almost entirely as high molecular weight forms. This is due the IGFs binding to a family of high affinity binding proteins (IGFBPs) which subsequently determine IGF bio-availability as well as modulating IGF biological activities.

Six distinct IGFBPs have been cloned and sequenced, all specifically binding the IGFs and having a limited affinity for insulin. The IGFBPs all share structural homology with their amino acid sequences at the amino- and carboxyl-terminal domains being highly conserved. This includes conservation of 18 cysteine residues in IGFBP-1 to IGFBP-5, with 12 residues in the amino-terminal region and 6 in the carboxyl-terminal. IGFBP-4 contains an additional two cysteine residues in the amino-terminal region while human and rat IGFBP-6 have only 16 and 14 cysteine residues respectively. This high cysteine conservation suggests disulphide bonding may be important in forming the specific high affinity IGF-binding site.

A brief overview of some features of the six IGFBPs will be given as reviewed by Cohick & Clemmons (1993), Langford & Miell (1993) and Rechler (1993).

IGFBP-1 has a molecular weight of 25 to 28 kDa and binds IGF-I and IGF-II with approximately equivalent affinities. It is abundant in human amniotic fluid and placental tissues with the liver considered the major source of production. In humans, IGFBP-1 is developmentally regulated and also shows diurnal variations.

IGFBP-2 is a 29 to 30 kDa non-glycosylated protein which has been purified from rat kidney and bovine liver cell lines and is the major binding protein in CSF and the central nervous system. This binding protein has a marked preferential affinity for IGF-II compared to IGF-I and is also developmentally regulated, being more abundant in rat fetal serum compared with adult serum.

IGFBP-3 is a 28 kDa protein that is *N*-glycosylated and forms molecular weight species of between 42 and 55 kDa. IGFBP-3 has a high affinity for both IGF-I and IGF-II and is the major circulating IGFBP in adult mammals, existing primarily as a high molecular weight (150 kDa) ternary complex. This complex is comprised of IGFBP-3, IGF-I or IGF-II and a third binding protein termed the acid labile subunit (ALS). ALS is a *N*-glycosylated protein of about 90 kDa which only binds IGFBP-3 in the presence of IGF-I or IGF-II. Because ALS is approximately 2-fold more concentrated than IGFBP-3 in plasma, the IGFBP-3/IGF complexes are primarily present as ternary complexes.

Less is known about the physiology of IGFBP-4, -5 and -6. IGFBP-4 is a 25 kDa protein that also exists as a *N*-glycosylated 29 kDa form. It binds IGF-I and IGF-II with approximately equal affinities and is second in abundance to IGFBP-3 in rat serum. This binding protein is also produced by cultured osteoblasts and other bone derived cell lines. IGFBP-5 is a 29 kDa non-glycosylated protein which preferentially binds IGF-II over IGF-I. Although it has been purified from human serum, it is present in low concentrations compared to the other IGFBPs. The kidney has been shown to express high levels of IGFBP-5 mRNA (Shimasaki *et al.*, 1991). IGFBP-6 is an approximate 22 kDa protein which has been purified from several human sources including serum and CSF. IGFBP-6 is glycosylated in samples from humans, and has increased affinity for IGF-II compared to IGF-I.

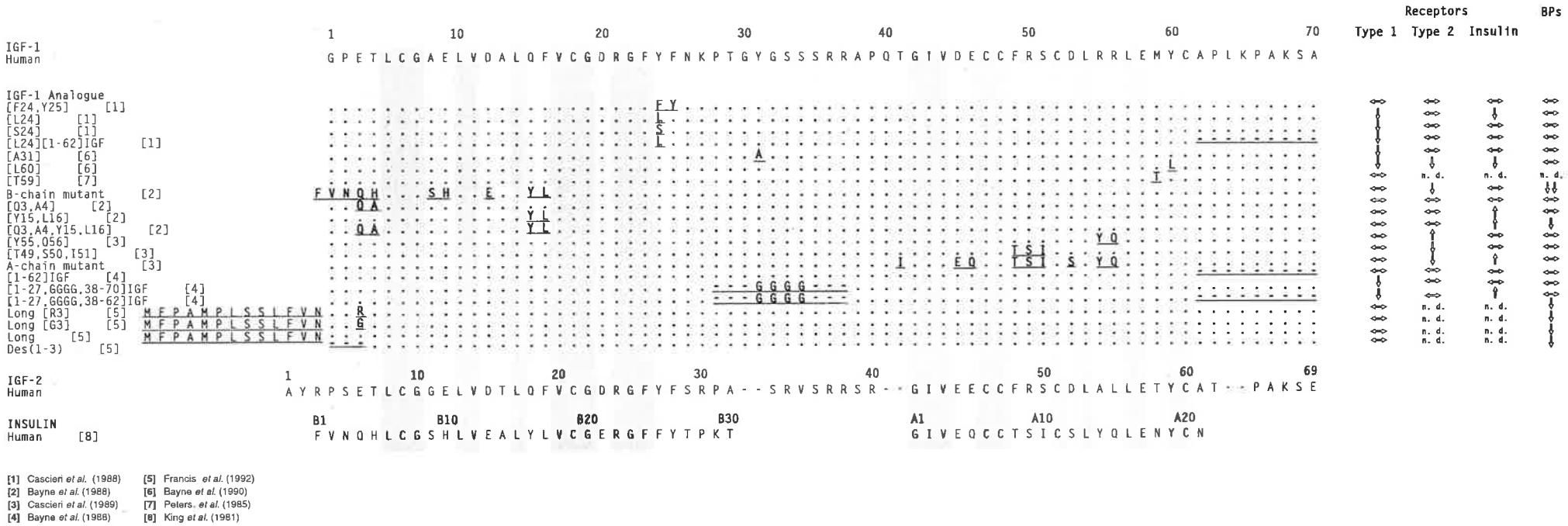
The IGFBPs have been shown to have several important roles that are essential to coordinating and regulating the biological activities of IGF-I and IGF-II. These involve increasing the circulating half-life of IGFs and thus regulating their metabolic clearance. They also mediate the transport of the IGFs in the serum and regulate the delivery to the vascular space and the extracellular fluids and further provide a specific tissue and cell delivery. The IGFBPs also modulate the affinity of the IGFs for the receptors and hence regulate metabolic and mitogenic effects at the cellular level. This modulation of IGF activity and cellular function has been studied extensively (reviewed by Sara & Hall, 1990; Cohick & Clemmons, 1993; Jones & Clemmons, 1995) and will not be covered further in this review.

As with the IGFs, the IGFBPs are hormonally, nutritionally and developmentally regulated (Cohick & Clemmons, 1993; McCusker & Clemmons, 1992; Thissen *et al.*, 1994). These aspects will also not be covered in this review except to acknowledge that these factors can affect IGFBP production and degradation as well as the binding affinity for the IGFs.

1.2.9 IGF analogues and structure-function studies

A large amount of research has been directed towards understanding the structure of the IGF molecule in relation to IGF function. This research has primarily attempted to determine which amino acid residues in the IGF molecule are involved in IGFBP and receptor interactions. These studies have used both naturally occurring variants as well as analogues generated by recombinant DNA technologies. Figure 1.4 shows amino acid sequences, compiled using data from some of the IGF-I analogues reported in the following section, and summarises their biological activities.

Several naturally occurring variant forms of IGF have been isolated. Des(1-3)IGF-I is an amino-terminal truncated form of IGF-I, lacking the first three amino acids, Gly Pro Glu. This variant has been isolated from bovine colostrum (Francis *et al.*, 1988), porcine uterus (Ogasawara *et al.*, 1989), human brain (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1989) and human platelets (Karey *et al.*, 1989). Precursor forms of IGF-I have been detected in plasma (Powell *et al.*, 1987) and conditioned media of cultured human



[1] Cascieri *et al.* (1988)
[2] Bayne *et al.* (1988)
[3] Cascieri *et al.* (1989)
[4] Bayne *et al.* (1988)
[5] Francis *et al.* (1992)
[6] Bayne *et al.* (1990)
[7] Peters. *et al.* (1985)
[8] King *et al.* (1981)

FIGURE 1.4
Comparative sequence data and bioactivity of insulin-like growth factor I and its analogues.
Dashed lines represent identical sequence compared to human IGF-I. Periods were used to frame shift peptides for maximal alignment between sequences. Complete sequences are given for other related molecules. Comparative receptor and IGFBP affinity data is summarised for the analogues where ascending and descending arrows, respectively, indicate increased and decreased affinity of greater than five-fold compared to IGF-I. No change in affinity is shown by the side-ways arrow. Not determined affinity is shown as n.d. (Adapted from Cascieri *et al.* (1991) and a diagram produced by G. Shooter, CRC for Tissue Growth and Repair, Adelaide, SA, Australia)

fibroblasts (Conover *et al.*, 1989). Several large molecular weight IGF-II variants have also been identified, from sources such as human serum (Zumstein *et al.*, 1985; Gowan *et al.*, 1987), CSF (Haselbacher & Humbel, 1982) and conditioned media (Yang *et al.*, 1985; Culouscou *et al.*, 1990). Cleavage variants of IGF-I and IGF-II have also been identified in human plasma (Jansen *et al.*, 1990).

1.2.9.1 Binding to type 1 IGF-receptors

Tyr24 in IGF-I has been identified as an important residue in high affinity type 1 receptor binding. Both the analogues [Leu24]IGF-I and [Ser24]IGF-I have reduced affinity for the type 1 IGF-receptor (Cascieri *et al.*, 1988). Similarly, Tyr27 in IGF-II, analogous to Tyr24 in IGF-I, has been found to be involved in IGF-II binding to the type 1 receptor (Beukers, *et al.*, 1991; Roth *et al.*, 1991; Sakano *et al.*, 1991). Furthermore, all three tyrosine residues (Tyr24, Tyr31 and Tyr60) of IGF-I have been implicated in type 1 receptor binding, as shown by their high protection against iodination in the IGF-I/receptor complex (Maly & Luthi, 1988) and by single amino acid substitutions at these residues decreasing receptor binding (Bayne *et al*, 1990a).

Other determinants in the C-domain are also thought to be involved in high affinity binding of IGF-I to the type 1 receptor. The analogue, [1-27, GGGG, 38-70]IGF-I, where the C-domain has been removed and a glycine bridge incorporated, caused a marked decrease in receptor affinity (Bayne *et al.*, 1989), part of which was due to the loss of Tyr31. Similarly, Tseng *et al.* (1987) chemically synthesised two chain molecules containing either the A and B domains of IGF-I and found a decrease in binding to the type I receptor, as did Slieker *et al.* (1994) when they prepared two chain IGF-I analogues by solid-phase peptide synthesis.

In contrast, large changes in the A, B and D regions of IGF-I have minimal effects on type 1 receptor affinity. The A chain mutant ([Ile41, Glu45, Gln46, Thr49, Ser50, Ile51, Ser53, Tyr55, Gln56]IGF-I) and the B chain mutant of IGF-I had minimal effect on type 1 receptor binding, but they resulted in a loss of type 2 IGF-receptor affinity (Bayne *et al.* 1988; Cascieri *et al.*, 1989). Removal of the D-domain, as in des(63-70)IGF-I,

similarly had a minor effect on type 1 receptor binding (Bayne *et al.*, 1989; Slieker *et al.*, 1994). However removal of the D-domain in IGF-II, des(62-67)IGF-II, had effects on both the type 1 and type 2 IGF-receptors leading to speculation that this region has determinants involved in receptor binding (Roth *et al.*, 1991).

Folding variants of IGF-I and IGF-II with mismatched disulphides have also been found to have reduced receptor binding as well as reduced binding to IGFBPs. This indicates the three dimensional structure of IGF-I, as maintained by the disulphides, is required for IGF function (Hodgkinson *et al.*, 1989; Oh *et al.*, 1991).

1.2.9.2 Binding to type 2 IGF-receptors

Analogues of IGF-I, substituted with analogous residues from the *A*-region of insulin, have altered affinity for the type 2 receptor but show limited effects on affinity for the type 1 receptor and serum IGFBPs (Cascieri *et al.*, 1989). The *A*-chain mutant and [Thr47, Ser50, Ile51]IGF-I showed decreased type 2 receptor binding whereas [Tyr55,Gln56]IGF-I has increased affinity (Cascieri *et al.*, 1989). Analogous residues in IGF-II have also been shown to be involved in type 2 receptor binding (Sakano *et al.*, 1991). These findings suggest that Arg55-Arg56 contribute to the relative low affinity of IGF-I for the type 2 receptor. Substitution at Tyr60 also results in a decreased affinity for type 2 receptor binding although this may be due to a conformational change or a destabilisation of the IGF-I molecule (Bayne *et al.*, 1990a).

The *B*-chain mutant had a 100-fold decreased affinity for the type 2 IGF-receptor (Bayne *et al.*, 1988). Further analogues with *B*-domain substitutions at Glu3, Thr4, Gln15 and Phe16 showed unaltered type 2 receptor binding, suggesting that either these two regions in the *B*-domain of IGF-I are not important for maintaining binding or that the more dramatic total change is required (Bayne *et al.*, 1988). As shown by the *B*-chain mutant, this region of IGF-I has distinct determinants which are required to maintain type 2 receptor binding, perhaps suggesting that residues Gly1, Pro2, Ala8, Glu9 and Asp12 could be involved.

Analogues prepared by Bayne *et al.* (1989) indicate that neither the *C*- nor *D*-domain is required for high affinity binding to the type 2 receptor.

1.2.9.3 Binding to IGFBPs

The *N*-terminal region of the IGFs has been shown to be important for binding to the IGFBPs. Des(1-3)IGF-I, a truncated form of IGF-I, has a greatly reduced affinity for the IGFBPs (Szabo *et al.*, 1988; Forbes *et al.*, 1988). Bagley *et al.* (1989) chemically synthesised amino-terminal truncated IGF-I analogues. They found deletions of three of four amino acid residues significantly decreased binding to IGFBPs secreted by L6 myoblasts. A further series of analogues with substitutions for Glu3, showed amino acids lacking negatively charged side chains resulted in reduced affinity for IGFBPs (King *et al.*, 1992). Similarly, Glu6 in IGF-II, analogous to the Glu3 residue in IGF-I, has been implicated in IGFBP interactions (Francis *et al.*, 1993).

The *B*-chain mutant and substitutions at residues Glu3, Thr4, Gln15 and Phe16 also resulted in a large decrease in affinity for serum IGFBPs (Bayne *et al.*, 1988) indicating that certain determinants within the *B*-domain are critical in maintaining high affinity binding to the IGFBPs. Further studies with purified IGFBPs have indicated that distinct regions of the IGF-I molecule are involved with the different IGFBPs (Forbes *et al.*, 1988; Clemmons *et al.*, 1990 & 1992a; Baxter *et al.*, 1992; Bach *et al.*, 1993; Oh *et al.*, 1993). For example the mutant [Tyr15, Leu16]IGF-I has a 10- and 100-fold reduced affinities for IGFBP-4 and IGFBP-5 respectively, but has limited reduction in affinity for IGFBP-1, -2 or -3 (Clemmons *et al.*, 1992a). Collectively these studies suggest that the amino-terminal residues of IGF-I contain the binding domain of IGFBP-3, while binding domains for IGFBP-1 and -2 are quite conserved and involve residues in the first helical domain of the *A*-region (Cascieri *et al.*, 1991). It has also been suggested that the binding specificities of IGFBP-4 and -5 are similar and may have larger binding pockets than those of IGFBP-1, -2, and -3 (Cascieri & Bayne, 1994).

1.2.9.4 Long-R^3-IGF-I

Within our group, recombinant IGF-I was originally produced by expression of a gene construct in Chinese hamster ovary cells (McKinnon *et al.*, 1991). Using this transformed mammalian cell system, IGF-I was secreted as a biologically active molecule into the extracellular medium. However with this method, the peptide yield was low and the production costs were high. Consequently, IGF-I was produced as a fusion protein in *Escherichia coli*, enabling production of large quantities of the peptide as inclusion bodies (King *et al.*, 1992). The fusion protein consisted of an amino-terminal extension peptide made up of the first 46 amino acids of methionyl porcine growth hormone ([Met^1]-pGH) followed by a Val-Asn dipeptide. With the *N*-terminal residue of IGF-I being Gly, the Asn-Gly sequence provided a hydroxylamine sensitive link between the [Met^1]-pGH(1-46) and the amino-terminal of IGF-I. Subsequently, a series of IGF-I fusion peptides were expressed which carried a shorter, 13 amino acid extension peptide (Francis *et al.*, 1992). Long-R^3-IGF-I is one of these fusion protein analogues (Figure 1.5). It is comprised of an IGF-I sequence, where Glu3 in the human IGF-I sequence has been replaced with Arg, the first 11 amino acids of [Met^1]-pGH, and the Val-Asn dipeptide (Francis *et al.*, 1992).

Amino-terminal analogues of IGF-I, such as long-R^3-IGF-I, show an increased biological potency *in vitro* which is due to changes in their affinities for the IGFBPs (Francis *et al.*, 1992). Although long-R^3-IGF-I has an approximate 3 to 4-fold reduced affinity for the type 1 IGF-receptor compared to IGF-I, it has very low binding to IGFBPs secreted by rat L6 myoblasts, having approximately 700-fold less affinity than IGF-I (Francis *et al.*, 1992). Consequently is a more potent stimulator of protein and DNA synthesis and inhibitor of protein degradation in L6 myoblasts, in comparison to IGF-I. However, in chick embryo fibroblasts, a cell line which does not secrete detectable IGFBPs into the extracellular medium, IGF-I is approximately 3-fold more potent than long-R^3-IGF-I in inhibiting protein degradation (Francis *et al.*, 1992).

In vivo, the growth responses observed in catabolic weight loss models such as induced diabetes and dexamethasone treatment in rats have shown that long-R^3-IGF-I is several fold more potent than IGF-I (Tomas *et al.*, 1992; Read *et al.*, 1991, 1992a & 1992b;

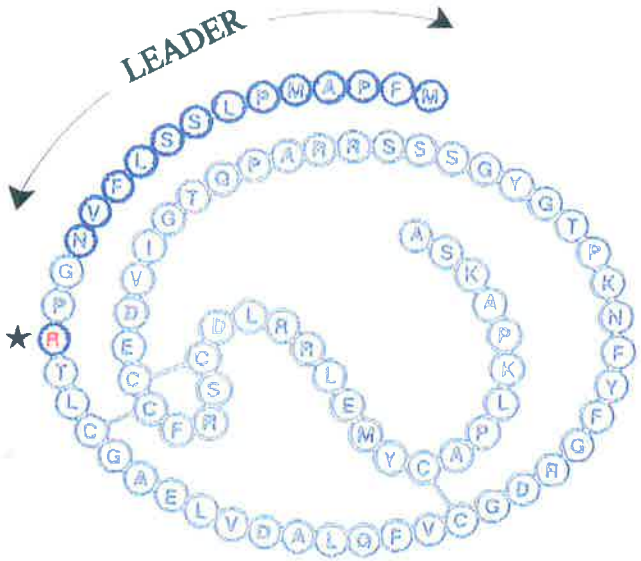


FIGURE 1.5
Schematic representation of the fusion protein, long-R^3-IGF-I. Long-R^3-IGF-I is IGF-I with a Glu3Arg mutation (indicated by the star) and a 13 amino acid N- terminal extension peptide (indicated 'Leader'). This extension peptide is comprised of a methionyl residue, the first eleven amino acids of porcine growth hormone and a redundant hydroxylamine-labile Asn-Gly bond. (Adapted from a diagram by B. Forbes, CRC for Tissue Growth and Repair, Adelaide, SA, Australia)

Section 1.2.10.3). This enhanced potency corresponds with the increased biological activity of long-R^3-IGF-I shown *in vitro* using cell cultures. As *in vitro* this effect has been attributed to the reduced affinity for IGFBPs, leading to a greater availability for receptor binding (Ballard *et al.*, 1987; Francis *et al.*, 1992) it has consequently been suggested that the greater biological potencies of the analogues *in vivo* is also a consequence of the greater proportion of peptide being available for interaction with cell receptors (Ballard *et al.*, 1991a & 1991b).

1.2.10 IGFs in the gastrointestinal tract

Recently, the gut has been shown to be a very sensitive target tissue for IGF-I (Read *et al.*, 1991 & 1992b), although this has not to date, been clinically tested. The presence of IGF-receptors has been shown in several species throughout the gastrointestinal tract. In addition, the gut has been shown to express IGF peptides, particularly in the fetal period, and IGFBPs. The recent availability of recombinant IGF has allowed research into the *in vivo* actions of the IGFs, however the effect of the gut as a target tissue has only been examined in a few studies.

1.2.10.1 IGF expression and localisation in gut

IGF mRNA and peptides have been detected throughout the human and rat gastrointestinal tract (D'Ercole *et al.*, 1986; Han *et al.*, 1987a & 1987b; Romanus *et al.*, 1988). The IGF peptides in the human fetal stomach and intestine have been localised to the epithelial cells, where greater concentrations are present in the villus compared to the crypt cells (Han *et al.*, 1987b).

Studies using assays of mRNA, show IGF-I and IGF-II mRNA are expressed particularly during the fetal and early neonatal period in the human and rat gastrointestinal tract (Han *et al.*, 1987a & 1988; Lund *et al.*, 1986; Lowe *et al.*, 1987; Hoyt *et al.*, 1988). In the human fetus, the stomach and intestine express higher levels of IGF-II mRNA compared to IGF-I mRNA (Han *et al.*, 1988). Furthermore, the IGF mRNAs are localised to mesenchymal tissue in the submucosa and lamina propria (Han *et al.*, 1987a).

In rats, IGF-I and IGF-II mRNA expression in the gastrointestinal tract is developmentally regulated (Lund *et al.*, 1986; Adamo *et al.*, 1989; Hoyt *et al.*, 1988). In the gastrointestinal tract of mature rats, IGF-I mRNA expression appears to be GH dependent (Hynes *et al.*, 1987; Lowe *et al.*, 1987) as well as regulated by nutrition (Lowe *et al.*, 1989).

There is little information regarding the IGF peptides in the gastrointestinal tissues of mature mammals, although IGF-I has been identified in a number of human exocrine secretions of the gastrointestinal tract including saliva, gastric juice, jejunal chyme, pancreatic juice and bile (Costigan *et al.*, 1988; Seidel *et al.*, 1989 & 1992; Chaurasia *et al.*, 1994). In comparison to IGF-I in serum, the IGF-I secreted into the gastrointestinal lumen is not bound to IGFBPs (Chaurasia *et al.*, 1994).

1.2.10.2 Presence of receptors and IGFBPs in the gastrointestinal tract

Studies have identified type 1 and type 2 IGF-receptors in the gastrointestinal tract of several species including pig (Schober at al., 1990), rat (Laburthe *et al.*, 1988; Young *et al.*, 1990; Ryan & Costigan, 1993), human (Korman *et al.*, 1989; Rouyer-Fessard *et al.*, 1990), rabbit (Pillion *et al.*, 1989; Termanini *et al.*, 1990) and specific intestinal cell lines (Culouscou *et al.*, 1990; Park *et al.*, 1990; Singh & Rubin, 1993). The receptors are widely distributed throughout the gastrointestinal tract (Laburthe *et al.*, 1988; Termanini *et al.*, 1990). In addition, the receptors show regional, cellular and tissue differences in IGF binding activity. For example, the colonic epithelium has greater binding than other regions of the gut (Laburthe *et al.*, 1988) while the intestinal epithelium expresses type 1 receptors that exhibit a villus tip-to-crypt gradient with greater numbers of receptors present in the crypt epithelial cells (Laburthe *et al.*, 1988). Other studies indicate differences in receptor distribution between the mucosal and muscularis layers (Termanini *et al.*, 1990). Muscular IGF-I binding was greater in the small and large intestine than in the oesophagus or stomach. Mucosal IGF-I binding was high in the stomach, which in the gastric fundus was further localised to the surface and mucous cells of the gastric epithelium (Termanini *et al.*, 1990). Type 1 and type 2 IGF-receptors can also show distributional differences. In human infants, type 1 receptors are expressed more densely in the muscularis than in the mucosa of the

jejunum, and within the mucosa the receptors are in greater densities in the lamina propria than in the surface epithelium (Heinz-Erian, 1992). In comparison, type 2 receptors are in greater densities in the mucosa than the muscularis, and are predominantly in the luminal part of the mucosa epithelial cells (Heinz-Erian, 1992).

There also appears to be a developmental change in receptor density, with higher levels of receptors present in fetal and neonatal gut compared to adult (Schober at al., 1990; Young *et al.*, 1990). The type 1 and type 2 IGF-receptors also undergo different patterns of change during post-natal development. In the rat, levels of type 1 receptors are maintained during suckling whereas type 2 receptor levels fall progressively (Young *et al.*, 1990).

Messenger RNA transcripts for several of the IGFBPs are expressed at high levels in various gut tissues (reviewed by Rechler, 1993). For example, in the fetal rat, IGFBP-1 and IGFBP-2 mRNA levels are abundant in stomach tissues, with lower levels in intestinal and in corresponding adult tissues. IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 mRNA have been reported in the adult rat stomach, small intestine and colon tissues. In addition, a mixture of IGFBPs are secreted from various intestinal-derived cell lines (Culouscou *et al.*, 1990; Park *et al.*, 1992; Oguchi *et al.*, 1993; Singh & Rubin, 1993).

It has been suggested that gut growth and function may be modulated by IGFBP production, as changes in levels of IGFBPs have been observed in rats with small bowel resections (Albiston *et al.*, 1992; Chen & MacDonald, 1992). Also the presence of significant concentrations of IGFBP-1, -2 and -3 in human and rat milk, indicates that the IGFs and the IGFBPs may play an important role in the development of the gastrointestinal tract (Baxter & Martin, 1989).

1.2.10.3 Action and effect of IGFs in the gastrointestinal tract

Selective gut growth in response to systemic IGF peptide administration has been reported in studies with normal rats as well as in animal models showing conditions of modified gut growth.

An early study in neonatal rats found IGF-I and IGF-II administration induced increases in brush border enzyme activities (Young *et al.*, 1990). More recent studies by Olanrewaju *et al.* (1992) and Seidel *et al.* (1992) indicate a trophic action of IGF-I as infusion of IGF-I directly into the ileal lumen of adult rats induced mucosal growth which was shown as an increase in mucosal wet weight, cellularity and total mucosal DNA, RNA and protein content.

Lemmey *et al.* (1991), demonstrated that subcutaneous administration of IGF-I, and des(1-3)IGF-I at a lower dose, produced beneficial effects in rats after surgical trauma associated with a 80 % jejunum-ileal resection. Treated rats showed improvements in weight gain and nitrogen balance, which were associated with marked increases in the organ weights of the gastrointestinal tract. These results were consistent with a similar study by Vanderhoof *et al.* (1992). Subcutaneous IGF-I administration has also been examined in other models of catabolic weight loss, such as glucocorticoid treatment and streptozotocin-induced diabetes. Tomas *et al.* (1992) found IGF-I and the two IGF-I analogues, des(1-3)IGF-I and long-R^3-IGF-I, partially reversed a catabolic state produced by dexamethasone treatment in rats, with marked improvements in whole body growth, nitrogen balance and protein turnover. The gut was observed as one of the most sensitive IGF target tissues, where IGF treatment groups were found to have a dose-dependent increase in gut weight, as a fraction of body weight, up to a maximal response of 45 % over the control animals. This effect was apparent in all regions of the gut examined, including the stomach, small intestine and colon. In the intestine, induced intestinal growth was predominantly through an increased cross-sectional tissue mass rather than an increased intestinal length (Read *et al.*, 1991, 1992a & 1992b). Similar results were obtained in a diabetic rat model where a dose-dependent stimulation of gut growth was observed for IGF-I and the more potent analogues, des(1-3)IGF-I and long-R^3-IGF-I, and this effect was not shown with insulin (Read *et al.*, 1991 & 1992b).

These studies all show that the gastrointestinal tract is a responsive target tissue for the IGF peptides and further suggests that these peptides may have clinical applications in a range of conditions.

1.2.11 Clinical applications of IGF

The recent increased availability of recombinant human IGF-I has made it possible to carry out studies *in vivo* to test the hypothesis that IGF-I can stimulate growth and exert insulin-like actions. Furthermore, it has also been possible to investigate whether this peptide can stimulate these functions in patients with growth disorders, catabolic conditions or diabetes (Froesch *et al.*, 1990; Cotterill, 1992; Laron, 1993; Clemmons & Underwood, 1994; Jones & Clemmons, 1995). In these clinical studies the administration of the IGF peptide has been via subcutaneous, intramuscular and intravenous routes.

The effects of IGF-I infusion (subcutaneous or intravenous) in normal human volunteers are comparable to the effects seen in rats (Table 1.2). Studies in healthy volunteers have demonstrated that administration of both GH and IGF-I have comparable anabolic and growth effects. IGF-I infusion, however, caused a decrease in hepatic glucose production and in circulating free fatty acids while increasing peripheral glucose uptake (Guler *et al.*, 1990a & 1990b; Boulware *et al.*, 1992; Turkalj *et al.*, 1992).

IGF-I treatment has been shown to have applications in the treatment of short stature due to GH insensitivity syndromes. These syndromes are characterised by complete or partial resistance to GH and a particular subtype is caused by an inherited defect in the GH receptor gene (Laron syndrome). Patients with Laron syndrome, commonly having short stature and slow growth, are clinically characterised as GH deficient despite increased serum concentrations of GH and abnormally low serum levels of IGF-I (Rosenbloom *et al.*, 1991). Clinical trials in children with this syndrome, show recombinant human IGF-I has a marked positive effect on linear growth (Wilton, 1992; Walker *et al.*, 1992). Similarly, poor linear growth associated with chronic liver disease and renal failure in children may also benefit from IGF-I therapy as children with these disorders have resistance to GH due to either low IGF-I production or high levels of IGFBPs (Blum *et al.*, 1991a & 1991b).

Studies in healthy volunteers showed IGF-I infusion caused decreased serum glucose and insulin levels, indicating the potential for IGF-I therapy in hyperglycaemic states such as diabetes mellitus (Boulware *et al.*, 1992; Morgan *et al.*, 1993; Sherwin *et al.*, 1994). IGF-I

Function	IGF-I effect
Glucose metabolism	↑ Glucose uptake ↓ Hepatic glucose production (human) ↑ Apparent insulin sensitivity Hypoglycemia (especially with intravenous administration)
Fat metabolism	↓ Serum ketones ↓ Serum free fatty acids (human) ↓ Triglycerides
Protein anabolism	↑ Protein synthesis ↓ Nitrogen excretion ↑ Total body protein accretion ↑ Body/organ weight especially of spleen, thymus, kidney and gut Improved wound healing
Renal function	↑ Glomerular filtration rate ↑ Renal plasma flow ↑ Speed of recovery from ischemic acute renal failure
Counterregulatory hormones	↓ GH ↓ Glucagon ↑ Catecholamines
Linear growth	↑ In hypopituitary and GH-insensitive subjects
Miscellaneous side effects	Parotid enlargement and tenderness, Bell's palsy, headache, peripheral edema, tachycardia.

TABLE 1.2
Effects of *in vivo* IGF-I administration to humans.
(From Jones & Clemmons, 1995)

treatment has been shown to be effective in some cases of diabetes, such as those resulting from mutations in the insulin receptor gene (Taylor *et al.*, 1990; Moller & Flier, 1991), due to hyperglycaemia being reduced through the IGF-receptor (Quin *et al.*, 1990; Schoenle *et al.*, 1991). IGF-I treatment may also be beneficial in cases where diabetes is associated with high titres of insulin-antibodies (Laron, 1993).

IGF-I treatment has been proposed as an anabolic therapy for patients with various catabolic conditions, including renal failure, corticosteroid therapy, protein wasting caused by malnutrition, burns and recovery from surgery or acute illness (Strock *et al.*, 1990; Cotterill, 1992; Miell *et al.*, 1992; Suh *et al.*, 1992; Clemmons & Underwood, 1994). Studies in normal volunteers in a catabolic state induce by fasting, showed IGF-I infusion caused an improvement in nitrogen balance (Clemmons *et al.*, 1992b; Young *et al.*, 1992) and a decrease in glucose and insulin levels (Clemmons *et al.*, 1992b; Morgan *et al.*, 1993). Other studies have also observed initial anabolic effects when IGF-I was infused into catabolic states as acquired by head trauma, immune deficiency disease and following major gastrointestinal surgery (Miell *et al.*, 1992; Clemmons & Underwood, 1994).

The responsiveness of the gut to IGF-I suggests clinical applications in the treatment of a range of gastrointestinal diseases and growth disorders (Table 1.3).

Short bowel syndrome is a malabsorptive disorder resulting from resection of a large percentage of the small intestine and patients consequently require parenteral nutrition. There is evidence that IGF-I and IGF-I analogues can improve intestinal regrowth and gut function following jejunal-ileal resection in rats (Lemmey *et al.*, 1991; Vanderhoof *et al.*, 1992; Section 1.2.10.3). Consequently, these peptides have therapeutic potential for improving intestinal growth and function in short bowel syndrome patients and possibly in the treatment of gut immaturity as found in premature infants. Similarly, IGF-I applications may be applied to conditions involving atrophy of the intestinal mucosa. Models of gut atrophy in rats, such as glucocorticoid treatment (Read *et al.*, 1991 & 1992a), drug induced chemotherapy conditions (Read *et al.*, 1994) and surface burns (Huang *et al.*, 1993), showed improvement in the recovery of intestinal mucosa due to IGF-I treatment. Furthermore, IGF-I infusion decreased bacterial translocation across the gut (Huang *et al.*, 1993) suggesting a reduction

Potential clinical applications for IGF-I in the treatment of gut diseases.

• Short bowel
• Atrophic gut
• Loss of barrier function
• Gut immaturity in the premature infant
• Radiation or chemotherapy-induced gut mucositis
• Inflammatory bowel disease
• Peptide ulcer disease

TABLE 1.3
Potential clinical applications for IGF-I in the treatment of gut diseases.
(Adapted from Read *et al.*, 1994)

in the loss of barrier function and possible clinical applications to reduce infection and sepsis. Conditions such as inflammatory bowel disease, may also be applicable to IGF-I treatment since patients with this disorder show an altered colonic mucosal morphology associated with poor nutrition and decreased growth rates in children, as well as decreased IGF-I levels (Kirschner & Sutton, 1986).

Peptic ulcer disease is a heterogeneous group of disorders related to an imbalance between acid-pepsin and mucosal resistance in tissues of the gastrointestinal tract (Grossman, 1980; Allen *et al.*, 1988). As ulcer healing involves a process of wound contraction and epithelialisation (Hase *et al.*, 1989), the ulcer bed may be subject to the same biological principles that govern the healing of deep external wounds. Although IGF treatment has not been investigated with respect to peptic ulceration, IGF-I is a major physiological mediator of normal wound healing (Lynch *et al.*, 1989; Steenfoe *et al.*, 1989; Skottner *et al.*, 1990). Several types of cells in the healing wound including macrophages (Rom *et al.*, 1988; Nagaoka *et al.*, 1990), fibroblasts (Spencer *et al.*, 1988), and platelets (Tokunaga *et al.*, 1990) release IGFs into the wound environment which can stimulate fibroblast replication (Conover *et al.*, 1983; Cook *et al.*, 1988), collagen deposition (Goldstein *et al.*, 1989; Steenfoe *et al.*, 1989) and possible endothelial cell migration (Grant *et al.*, 1987). Consequently, IGF-I may have potential to enhance re-epithelialisation in chronic gastroduodenal injury.

In conclusion, IGF-I administration has potential in the treatment of a number of clinical diseases and disorders. Furthermore, a convenient method of application of this peptide would be advantageous in the treatment of these conditions. Delivery of IGF-I by an oral means would be a convenient mode of administration.

1.2.12 Advantages of oral drug administration

There are several advantages for using orally delivered protein and peptide drugs. This method is a non-invasive route of delivery which also allows convenience in administration. Although oral administration in some cases may not be bio-equivalent to

invasive methods, the convenience to the patient can often outweigh the demand for the complete bio-equivalence. Furthermore, in the treatment of gastrointestinal disorders, oral therapeutics have additional advantages in providing a direct delivery to the gut as well as a reduction of side-effects in other tissues.

1.2.13 Evidence for oral activity of IGF-I

The expression of IGF-I mRNA and peptides in the tissues of the gastrointestinal tract (Section 1.2.10.1) suggests a direct action of IGF-I on the gut. Furthermore, this is also supported by the presence of type 1 IGF-receptors throughout the tract of several species (Section 1.2.10.2). IGF-I binding studies which show type 1 IGF-receptors are localised to the surface and secretory cells of the gastric epithelium in the rabbit stomach (Termanini *et al.*, 1990) which suggests these receptors can be activated by luminal growth factors and may function as transport epithelial proteins.

Oral activity of the IGF peptides is implicated in the growth and development of the gastrointestinal tract. Both IGF-I and IGF-II are present in amniotic fluid (Merimee *et al.*, 1984) which can be enterally assimilated in relatively large volumes by the fetus (Pritchard, 1966; Mulvihill *et al.*, 1985). Growth factors, including both IGF-I and IGF-II, have been reported in milk of various species thereby providing a source of IGFs to the suckling infant (Koldovsky & Thornburg, 1987; Grosvenor *et al.*, 1992). In addition, colostrum contains higher concentrations of these peptides (Koldovsky & Thornburg, 1987; Grosvenor *et al.*, 1992). For these milk-borne growth factors to influence neonatal development they would have to survive digestion in the infant tract. Although numerous factors influence the survival of these peptides in the gut (Britton & Koldovsky, 1989), the gastrointestinal tract of newborn mammals has been shown to have low proteolytic activity as well as increased permeability to proteins and other large macromolecules (Britton & Koldovsky, 1989). IGF receptors have been shown to undergo developmental regulation in the gut with higher levels being present in the fetal and neonate compared to the adult gastrointestinal tissues (Schober *et al.*, 1990: Young *et al.*, 1990). Furthermore, protease inhibitors have also been detected in the milk of several mammalian species including pig

(Weström *et al.*, 1982), rat (Rao *et al.*, 1993) and human (Lindberg, 1979). It is possible that these protease inhibitors may inhibit proteolysis in the mammary gland or the gastrointestinal tract, thereby facilitating the action or absorption of biologically important proteins.

Although the research has been very limited, oral activity of the IGFs is also suggested by studies involving direct intraluminal or oral administration of the IGFs in the gut. These studies have involved administration to both neonate and adult experimental animals.

Young *et al.* (1990) administrated IGF-I and IGF-II orally (1 μg/day for 6 days) to suckling rats and found jejunal brush border enzymes were stimulated but intestinal growth was not observed at this low dosage. Philipps *et al.* (1990) showed IGF-I could be taken up by the gastrointestinal tissues. ^{125}I-IGF-I, administered to suckling rats by stomach tube, was detected 30 min later in the stomach and intestinal wall with lesser amounts in the liver, lung, kidney and brain. Additional studies, with ^{125}I-IGF-I and ^{125}I-IGF-II, showed intact peptides were present in the stomach, intestinal wall and lumen with IGF-I approximately twice the concentration of IGF-II (Philipps *et al.*, 1992). Koldovsky *et al.* (1992) reported experiments using luminal contents from stomach, duodenum, jejunum, mid-jejunum and ileum of suckling (12 day old) rats. Incubation of ^{125}I-IGF-I and ^{125}I-IGF-II with luminal contents showed degradation of IGF-II, compared to IGF-I, was very low. In addition, the degradation of IGF-I and IGF-II was lower in the stomach, duodenum and ileum, compared to the jejunum and mid-jejunum. Grosvenor *et al.* (1992) cite several studies where dietary ^{125}I-labelled IGF-I was absorbed into the circulation of newborn calves, and also that colostrum-fed calves exhibit higher circulating IGF-I levels than milk-fed animals. Colostrum is reported having high levels of IGF-I compared to milk (Baxter *et al.*, 1984b; Francis *et al.*, 1986).

Studies which have investigated IGF-I and IGF-II stability in the adult gastrointestinal tract are very limited. Koldovsky *et al.* (1992), in addition to the neonate experiments, incubated ^{125}I-IGF-I and ^{125}I-IGF-II with luminal contents from adult (6 week old) rats. As in the suckling rats, IGF-II was more stable than IGF-I and both peptides showed decreased degradation in the stomach. A markedly increased degradation activity of IGF-I was shown

in the small intestinal luminal contents of adult rats. Experiments by Olanrewaju *et al.* (1992) and Seidel *et al.* (1992) report direct administration (by bolus injection and direct lumen infusion) of 10 nM IGF-I into the ileum of adult rats. Administration of the IGF-I to the site of action at a concentration of less than 1 % of that found in the rat gut, induced mucosal growth which suggested peptide stability. These experiments, however, appear incongruous with the reported rapid degradation of IGF-I in the lumen of the adult gut (Koldovsky *et al.*, 1992). They may reflect the prolonged fasting of the animals during the experiment resulting in decreased proteolytic enzyme secretion (Robberecht *et al.*, 1971; Hatch *et al.*, 1979; Rossi *et al.*, 1983) and hence increased peptide stability.

Studies by Xian *et al.* in progress at the start of this thesis work (and subsequently published as Xian *et al.*, 1995) have verified the rapid degradation of IGF-I in the stomach, small intestine and colon of the adult rat.

In conclusion, these studies suggest that although there is some evidence for bio-activity of oral IGF-I, oral applications of IGF-I appear to be limited, particularly in the adult gastrointestinal tract. For an orally administered peptide to be biologically active in the gut and thus be of physiological significance, it would have to survive the proteolytic activities in the lumen (Rao, 1991). A luminal growth factor may act by binding to specific receptors on the epithelial cells and exerting a biological activity, diffusing through the paracellular space to bind to specific receptors on the basolateral epithelial membranes, or the peptide may be absorbed by the epithelial cells in an intact biological form and then be transported across the basolateral membrane into the lamina propia thus gaining access to receptors on smooth muscle, neighbouring epithelial cells as well as transportation into the systemic circulation. However, if the gut is acting as a barrier to IGF-I action due to proteolytic degradation, then this is a clearly a major limiting factor to the oral activity of IGF-I. Consequently, it would be useful to design a gut-stable biologically active analogue of IGF-I.

1.2.14 Increasing peptide stability for oral therapeutics

A variety of methods have been used to increase the gastrointestinal stability of therapeutic peptides and proteins.

Co-administration of proteolytic inhibitors together with the oral peptide has been shown to increase peptide stability. Insulin has been shown to absorbed from the small intestine of mammals using *in vivo* and *in vitro* techniques in the presence of proteolytic inhibitors (Laskowski *et al.*, 1958; Kidron *et al.*, 1982; Ziv *et al.*, 1987). However, this method has not yet proved suitable for administration of oral therapeutics in reliable doses.

Controlled-release drug delivery systems via liposomes or polymers have been widely used to reduce sensitivity to protease inactivation and degradation by increasing the half-life of the protein or peptide (Langer & Moses, 1991; Ranade, 1991; Florence & Jani, 1994). These delivery systems release their contents by mechanisms involving diffusion, bioerosion or degradation, and swelling or generation of osmotic pressure. Examples of gastrointestinal delivery using these methods include capsule delivery of a prostaglandin E_1 analogue to the gastric mucosa for local treatment of gastric and duodenal ulcers (Oth *et al.*, 1992) and the use of polymeric matrices for insulin delivery (Langer *et al.*, 1985; Brown *et al.*, 1986b; Saffran *et al.*, 1986) as well as liposome-entrapped insulin (Arieta-Molero *et al.*, 1982; Hashimoto & Kawada, 1979).

Delivery of drugs by encapsulation methods, as described above, is useful in terms of avoiding specific regions of the gut but may not be effective for proteins, such as IGF-I, that are likely to be degraded rapidly on release from the delivery vehicle. Rather, structural modification to produce a gut-stable, yet bio-active protein would probably be required.

Absorption of peptides onto larger molecular weight protein has been shown to enhance gastric stability of peptides. Dextran sulphate has been shown to protect basic fibroblast growth factor (bFGF) from proteolytic degradation as well as heat and acid inactivation, due to the formation of a high molecular weight complex formed via ionic interactions close to putative heparin binding sites (Kajio *et al.*, 1992). Similarly, insulin absorbed to diazopolystyrene protects the insulin molecule from digestive enzymes *in vitro* and results in a hypoglycaemic response *in vivo* in rabbits (Shichiri *et al.*, 1971).

Structural modification of the protein by chemical or recombinant means have also improved stability and minimised enzymatic cleavage in some cases. Removal of protease-sensitive sites by site-directed mutagenesis was effectively used by Brinkman *et al.* (1992) to improve stability of exotoxin in the circulation of mice. Seno *et al.* (1988) found bFGF had enhanced stability in acid conditions and retained biological activity when cysteine residues were mutated to serine residues using site-directed mutagenesis. A similar result was also found by Caccia *et al.* (1992) after chemical modification of cysteine residues in recombinant human bFGF. Furthermore, a mutein protein which had serine residues substituted for the second and third cysteines in bFGF was found to promote healing of duodenal ulcers when oral administered orally to rats (Folkman *et al.*, 1991; Satoh *et al.*, 1991; Szabo *et al.*, 1994).

1.2.15 A gut stable IGF-I analogue?

To determine if a gut-stable bio-active analogue of IGF-I could be produced, one component of the gastrointestinal tract was selected for study.

In the gastrointestinal tract of adult mammals, digestion is initiated in the stomach where food is mixed with gastric secretions containing acid and gastric proteinases. The gastric contents are then intermittently discharged into the duodenum where the process of digestion continues with the secretion of pancreatic enzymes and bile salts. Proteins are hydrolysed to small peptides and free amino acids within the intestinal lumen and the enterocyte brush border (Gray & Cooper, 1971), then subsequently absorbed in the intestine (Grimble & Silk, 1989).

The pepsins are the predominant enzymes of the adult mammalian stomach. In comparison, the small intestine is a complex protease environment containing trypsin, chymotrypsin, elastase and carboxypeptidases as well as numerous proteases of brush border, cytoplasmic and lysosomal origin (Britton & Koldovsky, 1989). The proteases of the colon are in general undefined. This organ contains pancreatic enzymes, which are generally less abundant than in the small intestine, and several proteolytic enzymes of bacterial origin. It is unlikely that a completely stable analogue could be designed for all the regions of the gut. Consequently, the stomach environment was selected for design of a gut-stable bio-active

analogue of IGF-I. In addition to the stomach being less complicated than the other regions of the gastrointestinal tract due to the presence of only one major enzyme, pepsin, it is also an environment which has been widely examined. Furthermore, when a growth factor is administered orally this is the site of the first major proteolytic attack on the peptide by the gastrointestinal tract.

1.3 GASTRIC PROTEINASES

The gastric proteinases are found in the gastric juice of all vertebrates. These proteases are secreted as zymogens which are then converted to the active enzymes by the acidic conditions of the gastric juice.

1.3.1 Physiology of the stomach and gastric secretions

The gastric juice is a mixture of secretions produced by the gastric mucosa, including the surface epithelium and the various gastric glands in the stomach. The composition of the juice varies depending on the contributions of the various secreting structures. The major constituents of gastric juice are water, hydrochloric acid, and other electrolytes such as sodium, potassium, calcium, phosphate and bicarbonate. The major organic components include gastric proteinases, several types of mucus and various blood-group substances (Leach, 1961).

The mammalian stomach is usually divided into three main sections on the basis of the cellular constituents: the gastric cardia, gastric fundus and antrum. Gastric secretions enter the adult human stomach from the ducts of gastric glands. The glands in the gastric fundus (oxyntic glands) consist predominantly of three types of cells: mucous, chief and parietal cells. The parietal or oxyntic cells, found only in the glands of the fundus, are responsible for the production of hydrochloric acid. The chief cells, also called zymogen or pepsinogen cells, synthesise and secrete pepsinogens which are the zymogens of pepsin. Both these cell types also secrete several inorganic ions. Mucus, of complex composition, is secreted by the surface epithelial cells of the stomach, the chief cells of the oxyntic glands, and cells of the

cardiac and antral glands. The gastric mucins can contain A and H blood-group substance activities and possess intrinsic factor activity, promoting the absorption of vitamin B_{12}.

The proportions of the secretions can vary depending on the stimuli. Gastrin, produced by the antral mucosa, is a potent stimulant of acid production and acts synergistically with histamine and acetylcholine. Perfusion of the intestine with fat or HCl releases cholecystokinin and secretin respectively which inhibit gastrin release and thereby inhibit acid secretion (Said, 1992). Less is known about the secretion of the gastric proteinases. Secretion is affected by nervous and hormonal components that are closely related and may potentiate each other. Nervous stimulation is mediated by the vagus nerve which responses to smell, taste and swallowing of a meal. The vagal stimulation acts directly on the oxyntic cells as well as stimulating gastrin secretion. Gastrin also stimulates pepsinogen secretion which is proposed to occur through an osmotic influence on the gastric mucosa by the HCl (Foltmann, 1981).

In the course of a 24 h period, the adult human produces in the order of 2 - 3 litres of gastric secretions although after puberty, the rate of secretion declines with age. A fasting adult human stomach produces about 80 ml/hr of gastric secretions which on stimulation increases to approximately 110 ml/hr (Leach, 1961). The pH of gastric secretions varies widely from pH 1.1 to 8.4, however values of greater than pH 6 are usually indicative of gastric disorders. Fasting normal values in man are pH 2.2 - 2.7 with stimulated values of pH 1.2 - 2.0 (Leach, 1961).

1.3.3 Nomenclature

The gastric proteinases are broadly grouped into endopeptidases (or proteinases), which hydrolyse central peptide links in the protein molecule. All known gastric proteinases belong to the group EC 3.4.23, as described in the Enzyme Nomenclature recommended by the International Union of Biochemistry (Foltmann, 1981; Szecsi, 1992). As most of these enzymes have optimal activity under acid conditions they are designated acid proteinases, and due to the presence of two aspartic acid residues in the catalytic site they are also termed aspartic proteinases. The predominant gastric proteinases of adult mammals are designated

pepsin A (EC 3.4.23.1) and pepsin C (EC 3.4.23.3), with the trivial names of pepsin and gastricism respectively. Another group of gastric proteinases is chymosin or rennin (EC 3.4.23.4) which is associated with the development of neonatal mammals. Chymosin does not appear to be present in humans, however, a human pseudo-gene for chymosin has been reported (Örd *et al.*, 1990) and a protease resembling calf chymosin has been detected in gastric fluid from newborns (Henschel *et al.*, 1987). A fourth, poorly characterised group of minor components includes pepsin B (EC 3.4.23.3) which is found in pigs.

In non-stimulated human gastric secretions, the mean percentage of pepsin A and pepsin C is about 88 % and 10 % respectively (Jones *et al.*, 1993). Jones *et al.* (1993) also cite a bi-dimensional electrophoretic system which was developed for pepsin quantitation in gastric juice and which resolved up to 20 distinct forms of pepsin. These arise due to post-translational modifications of several gene products, emphasising the complexity of these enzymes (reviewed by Axelsson, 1992).

1.3.4 Enzyme structure and activation of zymogens

Most of the aspartic proteases are single chain enzymes consisting of approximately 350 amino acid residues and having a molecular weight of approximately 35 000. The three-dimensional structure of the enzymes indicate a bilobal molecule with a large cleft separating the two domains (Szecsi, 1992).

The zymogens for the gastric proteinases, pepsinogens, have a *N*-terminal pro-peptide of up to 50 amino acids long. These peptides, secreted from the chief cells in a neutral or slightly alkaline solution, are present in a stabilised, inactive conformation. H^+ ions initiate the formation of the fully active enzyme. At acidic pH, the zymogen molecules undergo a conformational change which results in enzymic activity without cleavage of a peptide bond. The zymogens can also be activated in an irreversible reaction which occurs due to limited proteolysis. This is an autocatalytic reaction, with the site of cleavage and the reaction pathway depending on the amino acid sequence of the zymogen, the specificity of the enzyme and on conditions such as the pH and concentration of zymogens and active enzymes. The cleavage reaction involves removal of an amino-terminal, basic peptide from the pepsinogen

molecule (Foltmann, 1981; Szecsi, 1992). Porcine pepsinogen at pH 2 for example, undergoes an initial cleavage which occurs between Leu^{18}-Ile^{19} resulting in an active intermediate pepsinogen which further requires another cleavage (Leu^{40}-Ile^{41}) to generate porcine pepsin A. The peptides which are generated during this activation of the pepsinogen are inhibitory to the action of porcine pepsin. At higher pH (3-4) activation predominantly occurs by removal of the entire pro-portion of the peptide as a single fragment.

1.3.5 Proteolytic activity

The gastric proteinases are difficult to characterise by a well defined optimum pH activity. The observed optima depend on the assay conditions, with factors such as the ionic strength of the buffers, the substrates and their extent of denaturation, and the duration of the experiment all affecting results. In general terms, it is recognised that the optimal proteolytic activity of pepsin A occurs at about pH 2 while pepsin C is characterised by a higher optima of approximately pH 3 (Foltmann, 1981).

Although differences are found between the gastric proteinases, they all rapidly lose activity in neutral or alkaline solutions by an irreversible deactivation. Human pepsin A, for example, is inactivated at pH 7 although pepsin C is relatively stable under these conditions (Walker & Taylor, 1979). Species differences also exist. The major pepsin in rat is stable at pH 7.5 compared to inactivation of porcine pepsin A at this pH (Furihata *et al.*, 1980).

Compared to other proteinases, on a molecular basis pepsin acts more slowly on proteins and still more slowly on peptides with the exact specificity of the enzyme being uncertain (reviews by Dixon & Webb, 1964; Tang, 1970; Fruton, 1970 & 1974; Foltmann, 1981). Study of the primary specificity of pepsin A involved a series of compounds of the type AX-YB, where the X-Y bond is cleaved. Pepsin exhibits a preference for Phe residues in the X position and a Tyr, Phe or Try residue at the Y position. Substitution on the aromatic ring prevents the action of the enzyme. Replacement of these aromatic amino acids by aliphatic residues, such as Leu or Met with hydrophobic side chains, markedly reduces the cleavage of the X-Y bond. Replacement of the X residue with Val or Ile completely inhibits the pepsin action due to the branching of the β carbon. Peptide bonds

containing Pro are not cleaved. Pepsin also exhibits an absolute requirement for the L enantiomer in both the X and Y positions. Apart from the amino acid residues adjacent to the hydrolysed peptide bond, residues which are two or three positions away from the cleaved peptide bond, also influence substrate binding and specificity. This is consistent with the proteinases having an extended active site (Visser *et al.*, 1976; Powers *et al.*, 1977; Kay & Dunn, 1992). The broad specificity of the pepsins are qualitatively quite similar although definite differences in the specificities of pepsin A and pepsin C have been demonstrated. For example, there are a total of 23 common cleavage sites on glucagon and oxidised ribonuclease A. In addition there are two specific pepsin A cleavage sites in oxidised ribonuclease A (Val-Ala and Phe-Val), and two pepsin C specific cleavage sites in glucagon (Tyr-Ser and Tyr-Leu) and ribonuclease A (Tyr-Ser and Tyr-Gln). On synthetic small peptides, pepsin C has a preferential hydrolysis of Tyr-Y bonds (Tang, 1970; Auffret & Ryle, 1979).

The aspartic proteases, including pepsin A, are strongly inhibited by pepstatins which are pentapeptides produced by various species of *Actinomyces*. Pepstatin also inhibits pepsin C, although this activity is approximately 100 times lower than that observed with pepsin A (Umezawa, 1976). Pepstatin is reported to act as a substrate-like inhibitor binding in the active site of the enzyme (Foltmann, 1981).

1.3.6 Pepsin and IGFs

The production of a pepsin stable IGF could have potential therapeutic applications in the stomach, and would also serve as a delivery system for intact passage to the duodenum. A few studies have used complete pepsin digestion to confirm the primary structure of the IGF-I and IGF-II. Although these studies have not described the kinetics of pepsin digestion, they do provide information regarding the pepsin-sensitive sites in the peptides.

A peptide map of purified and folded, recombinant human IGF-II was determined by Smith *et al.* (1989) using porcine pepsin digestion. The pepsin digestion was performed at room temperature in 10 mM HCl at an enzyme to substrate ratio of 25 : 1 (w/w) for 48 hours. The pepsin cleavage sites were determined by amino acid analysis and sequencing

of the isolated peptides by Edman degradation. They reported ten major pepsin cleavage sites with an additional five minor sites (Figure 1.6). Bayne *et al.* (1990b) used a similar approach to confirm the primary structure of porcine IGF-II. Conditions of pepsin digestion used were 2 hours at room temperature with an enzyme : peptide ratio of 1 : 100 (w/w) in 5 % formic acid. They reported the molecular mass of four digestion fragments, suggesting cleavage had occurred between residues Leu^{13}-Val^{14}, Gln^{18}-Phe^{19}, Tyr^{27}-Phe^{28}, and Leu^{55}-Leu^{56} as also obtained by Smith *et al.* (1989).

Forsberg *et al.* (1990) investigated modified variants of human IGF-I using porcine pepsin digestion. The peptides were digested at room temperature for 3 to 4 hours using a enzyme to substrate ratio of 1 : 20. In comparison to the IGF-II pepsin digestion, Forsberg *et al.* found six major and five minor cleavage sites (Figure 1.6).

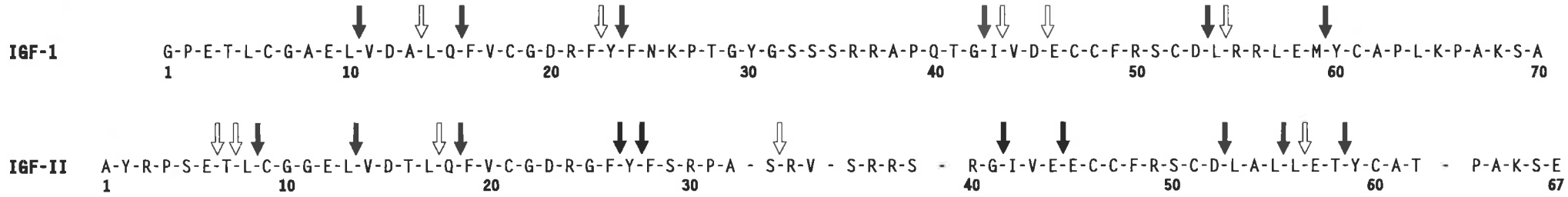


FIGURE 1.6
Primary sequence of IGF-I and IGF-II showing pepsin cleavage sites.
The major cleavage sites of pepsin are marked with filled arrows and the minor cleavage sites with open arrows.
(The diagram was constructed based on the work of Forsberg *et al.* (1990) on IGF-I and Smith *et al.* (1989) on IGF-II.)

1.4 AIMS OF THIS THESIS

At the commencement of this thesis a number of studies had indicated that IGF-I induced marked growth of the gastrointestinal tact when administered subcutaneously (Lemmey *et al.*, 1991; Read *et al.*, 1992a & 1992b; Vanderhoof *et al.*, 1992). The gut had been shown to be even more responsive to variants of IGF-I with reduced affinity for the IGFBPs, such as the *N*-terminally extended analogue, long-R^3-IGF-I (Read *et al.*, 1992a & 1992b; Tomas *et al.*, 1992). This increased biological activity is presumed to reflect the greater availability of the analogue for binding to the IGF-receptor (Francis *et al.*, 1992). IGF-I, however, had been shown to be rapidly degraded in all areas of the gastrointestinal tract (Koldovsky *et al.*, 1992) and therefore was likely to have limited therapeutic potential when administered orally. As IGF-I analogues with increased gut-stability have not been reported previously, the research in this thesis has attempted to design, produce and characterise IGF-I variants with increased stability to pepsin digestion while also retaining biological activity.

Pepsin was selected as the proteolytic enzyme against which to stabilise IGF-I for several reasons. This enzyme is located in the stomach which is the first major site of proteolytic degradation in the gastrointestinal tract. In addition, this region of the gut is less complicated than other regions in respect that the gastric proteinases are the predominant enzymes present in this organ. Although several isozymes of pepsin exist, these enzymes show a similar preference for proteolytic cleavage. Furthermore, pepsin A is the major protease of adult mammals.

Site-directed mutagenesis was selected as the method for producing IGF-I analogues. This approach has been effectively used to enhance stability of bFGF in the stomach environment (Seno *et al.*, 1988; Folkman *et al.*, 1991). Using this procedure, mutations can be directed at single amino acids which may also reduce the possibility of conformational changes that could adversely affect the biological activity of the peptide.

Long-R^3-IGF-I was used as the parent material for the variants due to its enhanced biological potency in the gastrointestinal tract compared to IGF-I. In addition, although

IGF-I had been shown to have limited stability in the gut, the effect of the *N*-terminal extension of long-R^3-IGF-I on gut stability was unknown. The presence of this extension peptide had potential to enhance the proteolytic resistance of the IGF-I peptide. Furthermore, as long-R^3-IGF-I is a fusion protein, it can be produced in larger quantities than the native molecule, IGF-I. This is due to the fusion partner ([Met^1]-pGH(1-11)-Val-Asn) which does not have to be cleaved and, in addition, facilitates the correct folding of the peptide (Francis *et al.*, 1992).

Specific aims of this thesis

1. To determine the initial cleavage sites of purified pepsin in long-R^3-IGF-I and assess whether the resulting cleavages affected biological activity.
2. To design and produce analogues of long-R^3-IGF-I which contain amino acid substitutions that enhance stability of the peptide to pepsin degradation.
3. To characterise the resulting analogues for pepsin resistance and retention of biological activity.
4. To assess the stability of the long-R^3-IGF-I analogues under *in vivo* conditions using luminal stomach flushings.

CHAPTER TWO

DIGESTION OF LONG-R^3-IGF-I BY PORCINE PEPSIN AND THE DESIGN OF PEPSIN RESISTANT ANALOGUES.

2.1 INTRODUCTION

IGF-I and the *N*-terminally extended variant, long-R^3-IGF-I (Francis *et al.*, 1992), have been shown to represent a potent class of growth factors for the entire gastrointestinal tract. (Read *et al.*, 1992; Steeb *et al.*, 1994). These responses suggest potential therapeutic applications for IGF-I and the analogues to stimulate gut growth and repair. However, both peptides are rapidly degraded in all areas of the gastrointestinal tract (Koldovsky *et al.*, 1992; Xian *et al.*, 1995) and are therefore likely to be of limited therapeutic value when administered orally. Delivery of the peptides by oral means would have advantages including convenience of application, direct delivery to the gut, and possibly reduced side-effects in other tissues. Since orally administered IGFs must first contend with proteolytic degradation by gastric pepsin, a pepsin-stable IGF could allow therapeutic applications in the stomach as well as intact passage to the duodenum. Gastrointestinal stability of therapeutics can be achieved by several methods. These include reducing sensitivity to protease inactivation by altering structure or use of a controlled-release drug delivery systems (reviews by Langer & Moses 1991; Ranade, 1991; Florence & Jani 1994)

In this study I have attempted to obtain a more pepsin-stable long-R^3-IGF-I peptide without substantial loss of bio-activity, by using site directed mutagenesis to alter the amino acid sequence at the most sensitive sites. The bonds susceptible to pepsin degradation in IGF-I have already been determined and include six major sites (Forsberg *et al.*, 1990; Figure 1.6). However the kinetics of the reaction are unknown, since Forsberg *et al.* (1990) identified the cleavage sites at only one time-point, representing an approximate 3 hour exposure to pepsin. In order to determine the relative susceptibility of these bonds, the time course of IGF degradation by pepsin needs to be determined. The first bonds liable to degradation would then become the primary targets for mutagenesis. Consequently, the first requirement was to determine which peptide bonds in long-R^3-IGF-I were most susceptible

to digestion by pepsin. Then, using that information, amino acid mutations would be selected for the most sensitive cleavage sites.

Several methods were used to assess the integrity of long-R^3-IGF-I after pepsin digestion. Reverse-phase high performance liquid chromatography (HPLC) was the primary technique, showing the digestion of the IGF-I analogue as the appearance of peptide fragments eluting at lower acetonitrile concentrations. The size of the peptide fragments was determined by electrophoresis on high density, SDS-polyacrylamide gels. *N*-terminal amino acid sequencing and mass spectroscopy identified the peptide fragments. In addition, IGF-receptor binding and protein synthesis stimulation assays were used to measure the retention of the receptor-binding site and biological activity respectively.

2.2 MATERIALS

Recombinant IGF-I and long-R^3-IGF-I (receptor grade) peptide were provided by GroPep Pty. Ltd., Adelaide, SA, Australia. Porcine pepsin A (EC 3.4.23.1) and Triton X-100 were purchased from Boehringer Mannheim Australia (Sydney, NSW, Australia). Bovine serum albumin (BSA: radioimmunoassay grade) and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) were purchased from Sigma, St Louis, MO, U.S.A. Carrier-free $Na^{125}I$ and [4,5-^{3}H]leucine were obtained from Amersham Australia Pty. Ltd., Sydney, NSW, Australia. All other reagents were of analytical grade.

Plastic ware for cell culture maintenance and assays was from Nunc, Roskilde, Denmark. L6 rat myoblasts (ATTC CRL 1458) were purchased from American Type Tissue Culture Rockville, MD, U.S.A. Hanks' balanced salts and fetal calf serum were purchased from Flow Laboratories, North Ryde, NSW, Australia. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco, Glen Waverly, NSW, Australia. Streptomycin and penicillin were obtained from Glaxo, Boronia, VIC, Australia.

HPLC equipment was from Millipore-Waters, Sydney, NSW, Australia and the reverse-phase microbore C4 column (2.1 x 100 mm) was purchased from Brownlee Laboratories, Santa Clara, California, U.S.A. HPLC grade acetonitrile (HiPerSolv™) was purchased from BDH Chemicals Ltd., Kilsyth, VIC, Australia and trifluoroacetic acid (TFA)

was from Fluka Chemie, Buchs, Switzerland. Milli-Q water was obtained using Milli-Q filtering apparatus (Millipore-Waters, Sydney, NSW, Australia) and solvents were filtered through 0.22 μm GV filters, also purchased from Millipore-Waters.

2.3 METHODS

All techniques were performed by myself unless otherwise indicated.

2.3.1 Long-R^3-IGF-I degradation with pepsin and subsequent HPLC analysis

Long-R^3-IGF-I (60 μg) was digested in 440 μl of 10 mM HCl using pepsin to peptide weight ratios varying from 1 : 10 to 1 : 1000 at 37° C. Degradation was stopped at different time points ranging from 15 to 240 min by adding 75 μl samples to 10 μl of 0.1 M NaOH followed by immediate storage at -20° C. The addition of sodium hydroxide increased the pH to greater than 8.0 thereby deactivating the enzyme and stopping peptide degradation (Furihata *et al.*, 1980). Prior to the addition of pepsin to the digestion reaction, a time zero degradation sample (10 μg) was taken and added to 10 μl of 0.1 M NaOH followed by storage at -20° C. Samples were analysed on reverse-phase HPLC using a microbore C4 column equilibrated with 0.1 % TFA in Milli-Q water. Separation was achieved using a gradient from 0 to 20 % acetonitrile over 5 min, then from 20 to 40 % acetonitrile over 40 min, using a flow rate of 0.5 ml/min at 30° C. Preliminary experiments showed this gradient, flow rate and column temperature resolved long-R^3-IGF-I and the initial peptide digestion fragments. The use of a controlled-temperature box, to standardise the column temperature, decreased the daily variation observed in peptide retention times. Peptide fragments were collected in siliconised tubes during the HPLC gradient, pooled then vacuum dried at room temperature. Between samples, the column was washed with 80 % acetonitrile.

To enable collection of sufficient concentrations of the peptide fragments for analysis by electrophoretic gels and biological assays, larger scale long-R^3-IGF-I digestions were performed as described above with the modification that the entire 60 μg sample of long-R^3-IGF-I was used per digestion time-point. In addition, the elution gradient was increased to 100 min between 20 to 40 % acetonitrile to further separate the peptide peaks

for collection. This corresponded to a 0.2 % per min acetonitrile gradient compared to a 0.5 % per min acetonitrile gradient in the small scale fractionation. The peptide fragments were then quantitated by analysing an aliquot on the original 0.5 % per min acetonitrile gradient.

The integrated peak area was converted to a protein concentration using a method based on Buck *et al.* (1989) where:

$$\mu g \text{ eluted protein}_y = AF_{215} / IF \times Q_{rel.y}$$

AF_{215} is the product of the integrated peak area at 215 nm and the flow rate, IF is the instrument factor and was taken as 1, and $Q_{rel.y}$ is a correction factor allowing for contributions of amino acid side chains to AF_{215} for protein$_y$. Q was defined as the extinction coefficient for the peptide at 215 nm divided by the molecular weight of the protein. Calculation of the extinction coefficient for long-R^3-IGF-I (Mr = 9111.1), as described by Buck *et al.* (1989), gave a value of 305 269 mol^{-1} cm^{-1} at 215 nm and thus a Q value of 33.50. The reverse-phase C4 column was standardised with long-R^3-IGF-I giving a linear response with increasing protein concentrations. Quantities of the peptide fragments were then subsequently estimated using the calculated Q value for long-R^3-IGF-I.

2.3.2 Standard protein techniques

2.3.2.1 Electrophoretic analysis of peptide fragments

Proteins were analysed on high-density polyacrylamide gels under non-reducing conditions on Pharmacia's "PhastSystem" (Pharmacia Biotech, Sydney, NSW, Australia) using the conditions and techniques suggested by the supplier. The gels were stained with Coomassie Brilliant Blue R2-250 by the "PhastSystem".

2.3.2.2 *N*-terminal protein sequencing

N-terminal amino acid sequencing was performed on a gas phase sequencer (model 470A; Applied Biosystems, Foster City, CA, U.S.A.) using the Edman degradation method as described by Hunkapiller *et al.* (1983). The peptide fragments were quantitated and vacuum dried in aliquots of approximately 250 pmoles. Peptide sequencing of the

samples was then performed by Miss. D. Turner, Department of Biochemistry, University of Adelaide, Adelaide, SA, Australia.

2.3.2.3 Determination of molecular mass

Molecular masses were determined by electrospray mass spectroscopy using a VG Biotech Quattro™ mass spectrometer (VG Biotech Ltd, Altrincham, Cheshire, UK). The peptide fragments were collected, quantitated and vacuum dried, then the mass quantitation was performed by Dr. M. Sheil, Department of Chemistry, University of Wollongong, Wollongong, NSW, Australia.

2.3.2.4 Iodination of IGF-I

IGF-I was iodinated with ^{125}I by the chloramine T method of Van Obberghen-Schilling and Pouyssegur, (1983) to specific activities between 60 and 80 Ci/g. The peptide (10 µg) was incubated with 1.0 mCi $Na^{125}I$ and 4 µg chloramine T, in a total volume of 130 µl for one minute. The reaction was stopped by the addition of 12 µg sodium metabisulphite, giving a final volume of 150 µl. The radiolabelled peptide was purified on a Sephadex G-50, 1 x 50 cm column, in 50 mM sodium phosphate, pH 6.5, 150 mM NaCl containing 0.25 % (w/v) BSA. The purified peptide was assayed for precipitability with 10 % (w/v) TCA and biological activity as described by Ballard *et al.* (1986). The IGF-I iodinations were performed by Mr. S. E. Knowles, CRC for Tissue Growth and Repair, Adelaide, SA, Australia.

2.3.3 In vitro assays

All cells were routinely grown at 37°C in 5 % CO_2 in DMEM containing 10 % fetal calf serum, 100 µg/ml streptomycin and 60 µg/ml penicillin. Routine maintenance and subculturing of cell lines was carried out by Miss. S. Tilley (CRC for Tissue Growth and Repair, Adelaide, SA, Australia). All assays were performed by myself except for the protein synthesis assay which was carried out by Mr. A. Talbot (CRC for Tissue Growth and Repair, Adelaide, SA, Australia).

2.3.3.1 Type 1 IGF-receptor binding

The method of Ross *et al.* (1989) was used to measure binding of the analogues to the type 1 IGF-receptor in rat L6 myoblasts. Confluent monolayers of L6 myoblasts in 24-place multiwells were washed with 2 x 0.5 ml of Hepes/ BSA buffer (0.1 M Hepes, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$ and 8 mM glucose, pH 7.6 containing 0.5 % (w/v) BSA) over a 2 h period at 4° C. Washing media was replaced with radiolabelled IGF-I (prepared as described in Section 2.3.2.4) and increasing concentrations of unlabelled peptides, in a total volume of 0.5 ml in Hepes/BSA buffer, and incubated at 4° C for 18 h. The samples of unlabelled peptides being tested were dried under vacuum then resuspended in 10 mM HCl and serially diluted using Hepes/BSA buffer. Following incubation, the cell monolayers were washed with Hanks' balanced salts at 0° C to remove unbound radioligand, then solubilised with 1 ml of 0.5 M NaOH containing 0.1 % (v/v) Triton X-100. Cell associated radioactivity was determined and binding was expressed as the percentage of bound radioactivity occurring in the absence of unlabelled peptide.

2.3.3.2 Stimulation of protein synthesis

Stimulation of protein synthesis by the growth factors was measured by the method of Francis *et al.* (1986) in L6 rat myoblasts. Confluent monolayers of L6 myoblasts were washed with 1 ml of serum-free DMEM for 2 h at 37° C. The washing media was then replaced with 900 µl of DMEM containing 1 µCi/ml [4,5-^{3}H]-leucine and 100 µl of sample. Peptide samples were prepared from material dried under vacuum, resuspended in 10 mM HCl, then serially diluted in 10 mM phosphate buffer, pH 7.4 containing 0.09 % (w/v) NaCl and 0.1 % (w/v) BSA. Cell monolayers were incubated at 37° C for 18 h, followed by washings with cold Hanks' balanced salts, cold 5 % (v/v) TCA, and finally Milli-Q water. The cells were solubilised with 1 ml of 0.5 M NaOH containing 0.1 % (v/v) Triton X-100 then 100 µl samples were added to scintillation fluid and cell associated radioactivity was determined. Protein synthesis stimulation was measured as the increased

[^{3}H]leucine into total cell protein over an 18 h incubation period compared with a buffer control.

2.4 RESULTS

2.4.1 Degradation kinetics of long-R^3-IGF-I using pepsin

The kinetics of digestion of long-R^3-IGF-I by pepsin were investigated in order to identify the most susceptible sites which would subsequently be the primary targets for mutagenesis.

Reverse-phase HPLC analysis of pepsin digestion at different time-points showed rapid breakdown of long-R^3-IGF-I to various peptide peaks (Figure 2.1). Using an enzyme to substrate ratio of 1 : 100 at 37° C, almost complete degradation of the long-R^3-IGF-I was obtained after 15 min (labelled A), resulting in the formation of two major peptides, labelled B and C in Figure 2.1. After a further 15 min digestion, two additional major peptide peaks, labelled D and E in Figure 2.1, were formed. As the pepsin digestion time increased, a number of minor peaks were noted which eluted from the reverse-phase C4 column at low acetonitrile concentrations. The same general pattern of long-R^3-IGF-I degradation was observed at each of the enzyme to substrate ratios examined. Figure 2.2 is an example of the degradation of long-R^3-IGF-I at varying enzyme to substrate ratios. In this figure, the HPLC traces show the digestion pattern of long-R^3-IGF-I after 30 min at pepsin to substrate ratios ranging from 1 : 10 to 1: 1000. The rate of long-R^3-IGF-I degradation increased with increasing pepsin concentration, as shown by the disappearance of peak A (Figure 2.2).

The major peaks, as indicated by peak area, were collected and then analysed by SDS-polyacrylamide gel electrophoresis. Figure 2.3 shows a non-reduced gel which has been Coomassie Brilliant Blue R-250 stained. Both the long-R^3-IGF-I standard (lane 1) and the long-R^3-IGF-I digested material which was collected from a time zero degradation analysis (lane 2) migrated on this gel with an approximate molecular mass of 10 500 Da under these conditions, higher than the electrospray molecular mass determination of 9 111 Da. Peptide fragment B (Figure 2.1) in lane 6 was shown to have a slightly lower molecular mass than the long-R^3-IGF-I parent material. This peptide ran at approximately 9 200 Da. The peptide

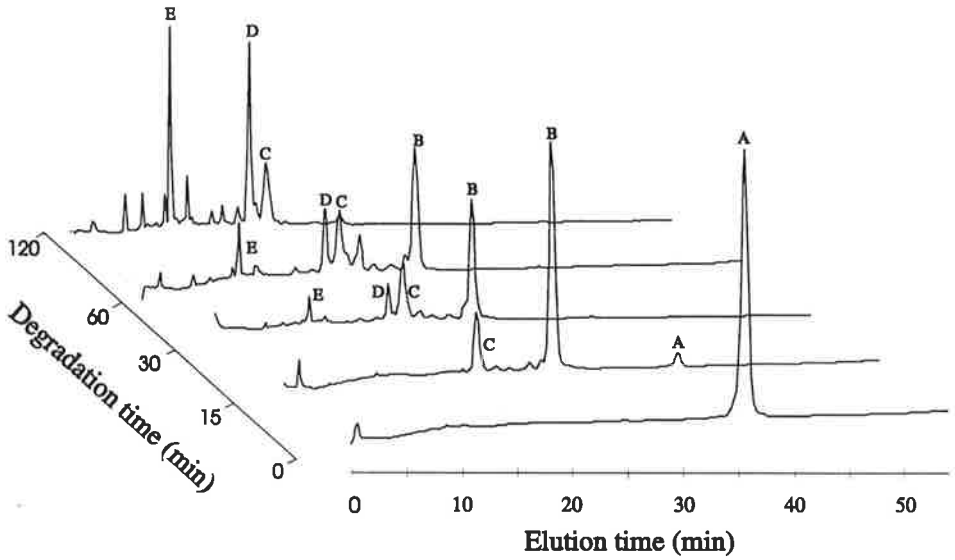


FIGURE 2.1
Time-course of long-R^3-IGF-I degradation by pepsin.
Degradation of long-R^3-IGF-I was determined using an enzyme : substrate ratio of 1 : 100 (w/w) at 37° C. At different time points the digestion was stopped by the addition of 0.1 M NaOH and the reaction analysed by reverse-phase HPLC on a microbore C4 column, using a gradient of acetonitrile in 0.1% (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at A_{215}.
Labelling of the peptides is as follows :
A , the parent molecule, long-R^3-IGF-I; B , FVN-R^3-IGF-I; C , the 10 amino acid extension peptide fragment; D and E , peptide fragments as indicated in Figure 2.4.

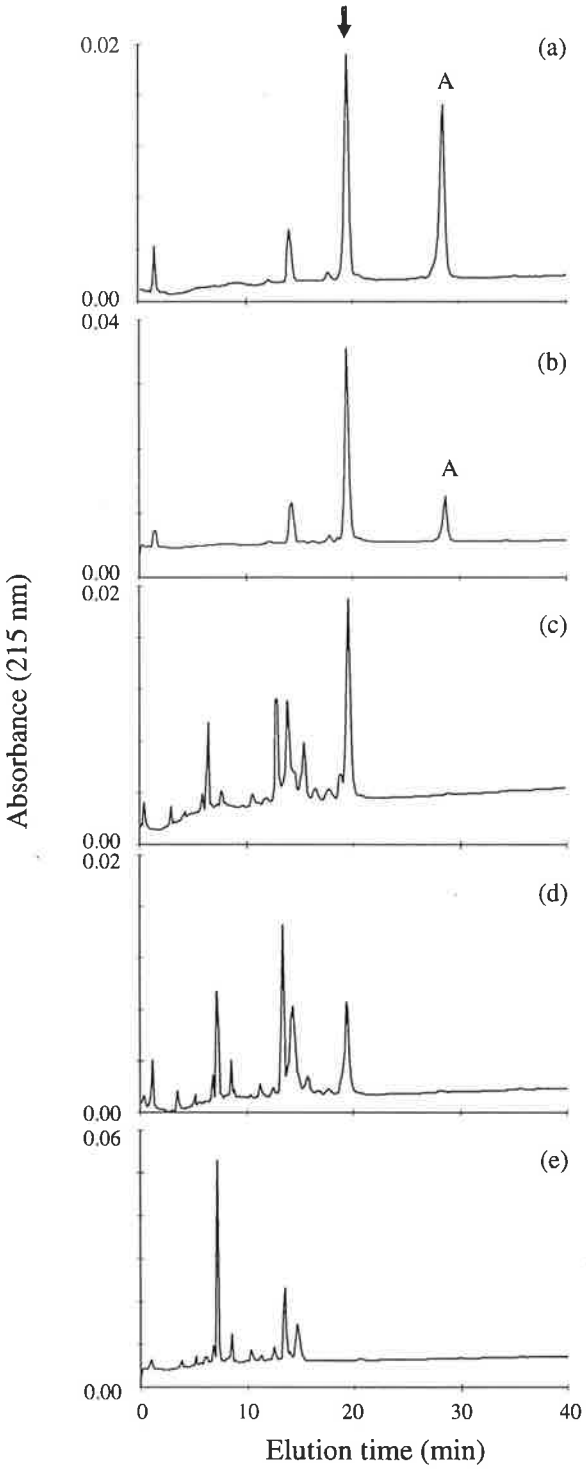


FIGURE 2.2
Effect of enzyme : substrate ratio on pepsin degradation of long-R^3-IGF-I, as analysed by reverse-phase HPLC.
Degradation of long-R^3-IGF-I was performed using a pepsin : substrate (w/w) ratio of (a) 1 : 1000 (b) 1 : 300 (c) 1: 100 (d) 1 : 30 and (e) 1 : 10 at 37° C. The 30 min digestion time-points were analysed by HPLC on a Brownlee Aquapore C4 column using a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at A_{215}. Long-R^3-IGF-I is labelled A and the arrow indicates the FVN-R^3-IGF-I peptide.

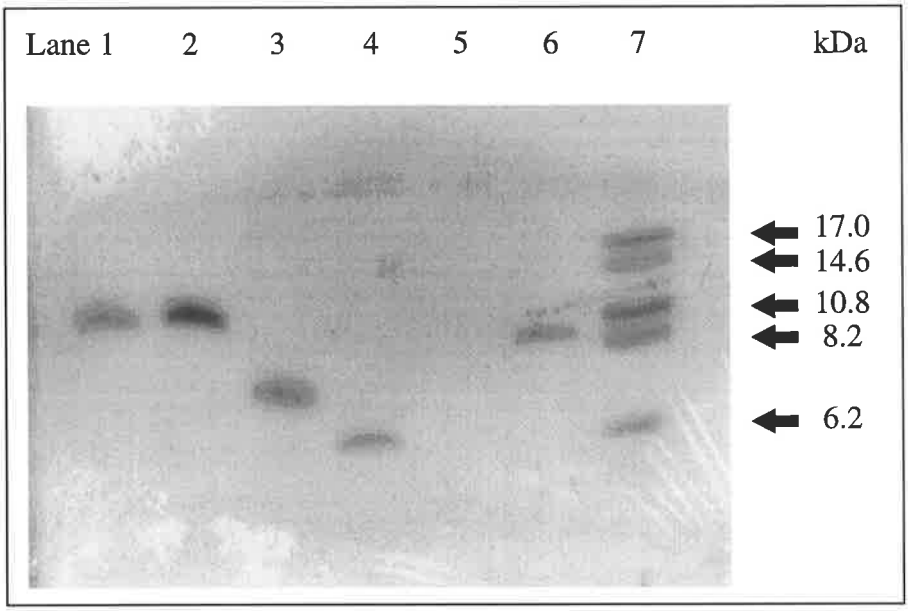
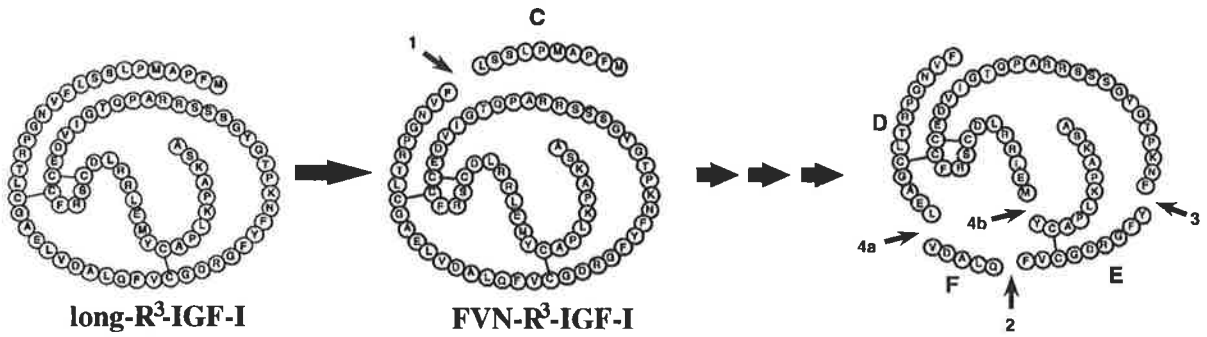


FIGURE 2.3
Polyacylamide gel analysis of pepsin digestion fragments of long-R^3-IGF-I. Long-R^3-IGF-I, digested with pepsin at 37° C, was analysed by HPLC on a Brownlee Aquapore C4 column using a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. Peptide peaks were collected, pooled, and dried then electrophoresed on a high density SDS-poyacrylamide gel under non-reducing conditions. The proteins electrophoresed were: Lane 1, long-R^3-IGF-I standard; Lane 2, Long-R^3-IGF-I collected from a time zero degradation point; Lane 3, 4, 5, and 6 are peptides D, E, C and B, respectively, as indicated in Figure 2.1 and Figure 2.4. Molecular weight markers are in lane 7 with the arrows indicating the molecular weights of the markers in kDa.



Peptide	Molecular mass (Da) Calculated	Actual
B = FVN-R^3-IGF-I	8037.3	8037.3
C	1093.5	1093.8
D	5354.8	5354.5
E	2210.0	2209.5

FIGURE 2.4
Kinetics of pepsin digestion on long-R^3-IGF-I.
Schematic representation showing the kinetics of pepsin digestion on long-R^3-IGF-I to the resulting peptide fragments. Sites of cleavage are numbered. Initial cleavage is at the Leu10-Phe11 bond in the *N*-terminal extension peptide to form FVN-R^3-IGF-I and a 10 amino acid peptide, labelled C. The second and third cleavage sites are at the Gln15-Phe16 and Tyr24-Phe25 bonds respectively in the IGF-I moiety. Cleavage then occurs at two further sites, Leu10-Val11 and Met59-Tyr60 marked 4a and 4b respectively, to produce three peptide fragments labelled D, E, F. The electrospray mass spectrometry values of the fragments are shown with their calculated values, based on a molecular mass of 9111.1 for long-R^3-IGF-I.

peaks D (lane 3) and E (lane 4), as indicated in Figure 2.1, were shown to be smaller peptide fragments than peptide B with molecular masses of about 6 600 and 5 800 Da respectively. The peptide fragment labelled C in Figure 2.1 was not detected using these electrophoresis conditions (lane 5). During the large scale collection of long-R^3-IGF-I digestion fragments, two additional peaks which eluted at acetonitrile concentrations between that of fragment C and B were also collected and subjected to non-reducing SDS-polyacrylamide gel electrophoresis. These peptides migrated with molecular masses of slightly lower than fragment B though higher than fragment D. This suggests these peptide peaks may be intermediate digestion fragments perhaps due to the removal of a small peptide, such as fragment F in Figure 2.4. As these peptide peaks represented small amounts of peptide, further analysis was not possible. The presence of these peptides at low concentrations under these digestion conditions suggest they are rapidly degraded to smaller peptide fragments.

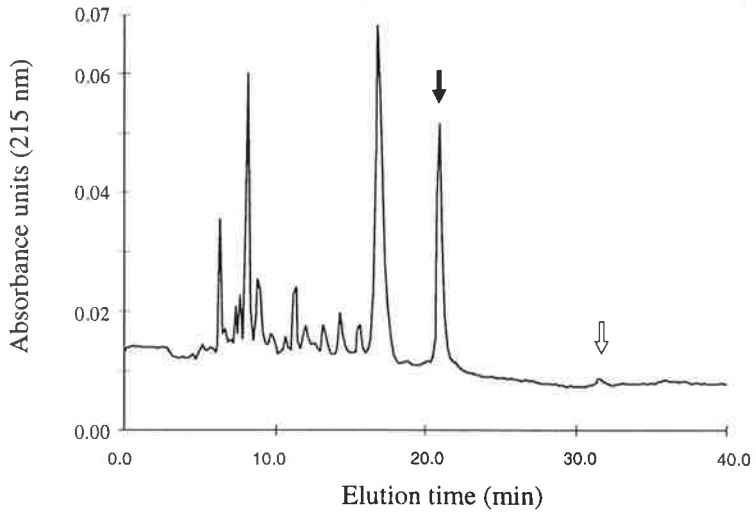
Further collection of the major peaks (labelled B, C, D, and E, Figure 2.1) followed by a combination of *N*-terminal sequencing and mass spectrometry showed long-R^3-IGF-I to be degraded to the peptide fragments indicated in Figure 2.4. The first cleavage site observed was between Leu10-Phe11 in the *N*-terminal extension peptide. This cleavage resulted in the formation of a 10 amino acid peptide (labelled C, Figure 2.1 and Figure 2.4) and a peptide subsequently termed, FVN-R^3-IGF-I (labelled B, Figure 2.1 and Figure 2.4). After this initial cleavage, FVN-R^3-IGF-I was then rapidly proteolysed at four other sites to produce three fragments (labelled D, E and F, Figure 2.4). These cleavage sites were between amino acid residues: Leu10-Val11, Gln15-Phe16, Tyr24-Phe25 and Met59-Tyr60 respectively in the IGF-I moiety.

The relative susceptibility of the four cleavage sites in the IGF-I moiety (Leu10-Val11, Gln15-Phe16, Tyr24-Phe25 and Met59-Tyr60) was suggested by the following results. The second cleavage site was determined at the Gln15-Phe16 bond (labelled 2 in Figure 2.4) due to the enhanced pepsin-stability of the long-R^3A^{16}-IGF-I analogue (refer to Chapter 3). The third most susceptible pepsin-cleavage site was indicated as Tyr24-Phe25 (labelled 3, Figure 2.4). This was determined by estimating protein amounts following *N*-terminal amino acid sequencing of the complete digestion mixture of

long-R^3A^1-IGF-I. Subsequent cleavage then occurred at the bonds Leu10-Val11 and Met59-Tyr60 in the IGF-I molecule (labelled 4a and 4b respectively, Figure 2.4).

The analogue, long-R^3A^{16}-IGF-I (refer to Chapter 3) was digested by pepsin for 120 min in 10 mM HCl at an enzyme to substrate ratio of 1 : 30. On termination of the degradation reaction the complete digestion mixture was *N*-terminal amino acid sequenced to determine the pepsin cleavage sites. The amino acid sequencing data indicated digestion fragments with amino-terminal sequences, Phe-Val-Asn-Gly-Pro, Met-Phe-Pro-Ala-Met and Phe-Asn-Lys-Pro-Thr in a concentration ratio of approximately 55 : 44 : 1. These sequences correspond to pepsin cleavage sites at positions 1 and 3 in Figure 2.4. A sample from a more complete pepsin digestion of long-R^3A^{16}-IGF-I, 40 min at an enzyme to substrate ratio of 1 : 30, was also analysed using amino acid sequencing. Following termination of the degradation reaction, by the addition of NaOH and freezing, an aliquot was analysed by reverse-phase HPLC and the remaining digestion mixture was *N*-terminal amino acid analysed. Figure 2.5 shows the peptide digestion pattern as determined by reverse-phase HPLC and a schematic representation indicating the sites of pepsin cleavage in long-R^3A^{16}-IGF-I as determined using the amino acid sequencing data. The HPLC analysis shows that at this time-point the intact long-R^3A^{16}-IGF-I peptide is present in very limited quantities, as indicated by the open arrow. However, FVN-R^3A^{16}-IGF-I (as shown by the filled arrow) was still apparent in significant concentrations as it represented 17 % of the total digestion peak area. In addition to the FVN-R^3A^{16}-IGF-I fragment, numerous other cleavage fragments were present as shown by the series of peaks eluting in lower acetonitrile concentrations. Analysis of the *N*-terminal amino acid sequencing data suggests that pepsin cleavage has not been completely inhibited at the Gln15-Ala16 bond since the amino acid sequence Ala-Val-X-Gly-Asp was observed, where X represents a cysteine residue. Pepsin cleavage is also indicated at the Leu10-Val11, Tyr24-Phe25, Met59-Tyr60 bonds, three of the four other initial cleavage sites determined in long-R^3-IGF-I. Other bonds that were susceptible to pepsin cleavage were the 'major' cleavage sites Gly42-Val43 and Asp53-Leu54 identified by Forsberg *et al.* (1990) as well as the 'minor' cleavage site Phe23-Tyr24.

(a)



(b)

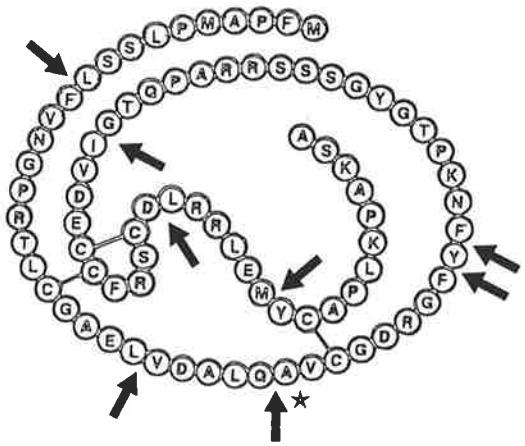


FIGURE 2.5
Pepsin digestion of long-R^{3}A^{16}-IGF-I.
(a) Pepsin digestion of long-R^{3}A^{16}- IGF-I as analysed by reverse-phase HPLC. Digestion occurred for 40 min at 37° C using an enzyme to substrate ratio of 1 : 10. The HPLC gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. Protein elution was monitored at A_{215}. The filled and open arrows indicate the elution position of long-R^{3}A^{16}-IGF-I, and the digestion fragment, FVN-R3A^{16}-IGF-I, respectively.
(b) Schematic representation of the pepsin cleavage sites in long-R^{3}A^{16}-IGF-I as indicated by *N*-terminal amino acid sequencing of the degradation mixure. The point mutation is labelled with the star.

2.4.2 Biological activities of long-R^3-IGF-I peptide fragments

The major peptide fragments were assessed for their IGF type 1 receptor binding as measured in rat L6 myoblasts (Figure 2.6). FVN-R^3-IGF-I had an approximate two-fold higher affinity for the type 1 receptor than long-R^3-IGF-I with half-maximal responses determined at 5.8 ng/0.5 ml and 12.5 ng/0.5 ml of added peptide, respectively. The other peptide fragments, (peptides C, D and E : Figure 2.4) had no measurable binding to the IGF-receptor at values between 2.5 ng and 100 ng of added peptide. Since FVN-R^3-IGF-I bound to the type 1 IGF-receptor, this peptide was also assessed for biological activity as measured by stimulation of protein synthesis in rat L6 myoblasts. At the two concentrations of peptide measured (1.3 and 13 nM), FVN-R^3-IGF-I gave maximal stimulation of protein synthesis. This showed the FVN-R^3-IGF-I peptide was biologically active, and had an activity between that of IGF-I and long-R^3-IGF-I.

2.5 DISCUSSION

2.5.1 Pepsin cleavage sites in long-R^3-IGF-I

Pepsin cleavage sites were characterised in the long-R^3-IGF-I growth factor as this peptide is more potent than the parent material in the gastrointestinal tract (Read *et al.*, 1992; Steeb *et al.*, 1994). IGF-I had been shown to have a limited half-life in all areas of the gastrointestinal tract (Koldovsky *et al.*, 1992) although the effect of the *N*-terminal extension peptide in long-R^3-IGF-I on gut stability was unknown. The initial pepsin cleavage sites in long-R^3-IGF-I were identified as the Leu10-Phe11 bond in the *N*-terminal extension peptide followed by cleavage at Leu10-Val11, Gln15-Phe16, Tyr24-Phe 25 and Met59-Tyr60 respectively in the IGF-I moiety. The rapid cleavage observed at Leu10-Phe11 in the *N*-terminal extension peptide, indicated a limited stability advantage for long-R^3-IGF-I compared to IGF-I in the gastrointestinal tract. This finding is consistent with recent work by Xian *et al.* (1995) where time-course degradation studies with rat stomach, duodenum, ileum and colon luminal flushings, showed long-R^3-IGF-I and IGF-I had virtually identical half-lives in all four gut regions, demonstrating the extension peptide did not protect native IGF-I

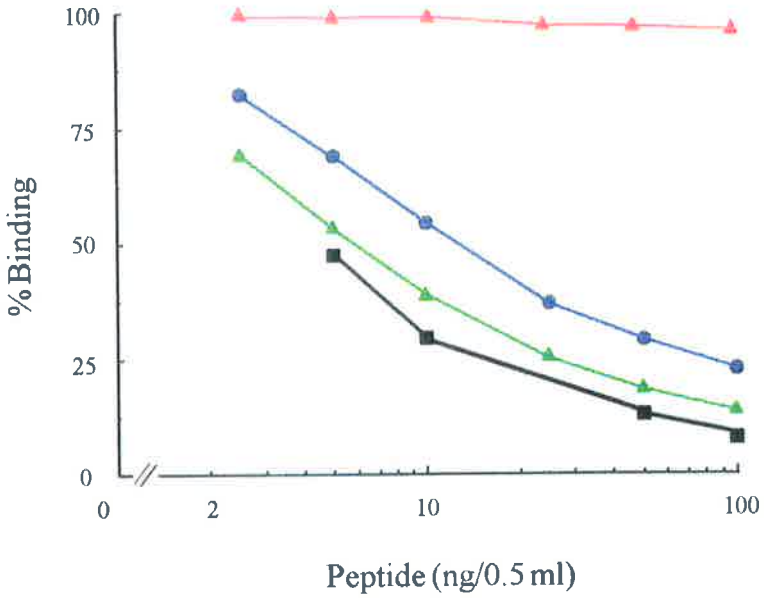


FIGURE 2.6
Binding of the major pepsin long-R³-IGF-I cleavage fragments to type 1 IGF-receptors on rat L6 myoblasts.
The peptides tested were: human IGF-I (■)
long-R³-IGF-I (●)
FVN-R³-IGF-I (▲)
peptides C, D, and E as indicated in Figure 2.4 (▲)

from degradation. In light of my results, this can be explained for the stomach environment, as the first site of pepsin cleavage occurs in this extension peptide.

Pepsin has a preference for cleavage between two adjacent hydrophobic residues (especially Phe) but it also hydrolyses proteins at a wide range of peptide bonds, typically those involving aromatic or large aliphatic residues (reviewed Dixon & Webb, 1964; Tang, 1970; Fruton, 1971 & 1974; Foltmann, 1981). In addition, the sequence around the cleavage bond also exerts a strong effect upon the rate of hydrolysis (Fruton, 1974), presumably reflecting the extended binding site of the protease (Powers *et al.*, 1977; Kay & Dunn, 1992). Of the cleavage sites I observed in long-R^3-IGF-I, four of these bonds (Leu10-Phe11 in the *N*-terminal extension peptide, and Gln15-Phe16, Tyr24-Phe 25 and Met59-Tyr60 in the IGF-I moiety) contained an aromatic residue in the P'_1 position* which is particularly favourable for hydrolysis by pepsin (Trout & Fruton, 1969). Furthermore, the Tyr24-Phe25 bond contains hydrophobic amino acids at both the P_1 and P'_1 positions. Although the Leu10-Val11 bond, which I also found to be susceptible to pepsin cleavage, does not contain an aromatic amino acid, pepsin is known to hydrolyse substrates containing Leu residues (Fruton, 1970). In addition, the analogous leucyl-valine link in the *B*-chain of insulin has also been shown to be susceptible to pepsin cleavage (Dixon & Webb, 1964).

Although the amino acid preferences of pepsin would be a major determinant of the order of cleavage in the long-R^3-IGF-I molecule, the secondary and tertiary structure of the peptide would also influence the availability of these cleavage sites and hence the rate of hydrolysis. Presumably, the sites most susceptible to pepsin cleavage are on the surface of the long-R^3-IGF-I molecule. The rapid degradation of long-R^3-IGF-I that was observed at the Leu10-Phe11 in the *N*-terminal extension peptide suggests this site is readily accessible for pepsin proteolysis. Preliminary NMR data (personal communication, Dr J. A. Carver, University of Wollongong, NSW, Australia) indicates the extension peptide has a mobile random structure. As the extension peptide is a flexible region with putative sites exposed,

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* The specificities of proteases are often characterised by the amino acid residues adjacent to the hydrolysed peptide bond (Schechter & Berger, 1967). These residues are designated as P_4-P_3-P_2-P_1-P'_1-P'_2-P'_3-P'_4 where the hydrolysed bond is P_1-P'_1.

this most likely accounts for its rapid pepsin cleavage. The IGF-I moiety of long-R^3-IGF-I has a more defined structure (Cooke *et al.*, 1991; Sato *et al.*, 1992 & 1993) suggesting that pepsin cleavage in this region would be less rapid with the cleavage sites reflecting their relative accessibility to the enzyme. The finding of the Gln15-Phe16 bond as the next most susceptible cleavage site could reflect its positioning at the end of a α-helix which may be in an area of greater flexibility and therefore increase the exposure of the peptide bond to pepsin. Although the Tyr24-Phe25 bond is in a relatively defined area of secondary structure (an extended conformation), also indicated by this region being involved in receptor binding, this peptide bond may have an increased susceptibility to cleavage due to the presence of the two hydrophobic residues. This may also apply to the Met59-Tyr60 bond. The adjacent amino acid (Cys61) is involved in a disulphide bond which would impart a degree of constraint on this area while the presence of a Met at the P_1 position increases the susceptibility of the site to pepsin cleavage (Dixon and Webb, 1964). The Leu10-Val11 bond is in a well defined area of secondary structure, an α-helical domain of the IGF-I molecule. Although Leu bonds are hydrolysed by pepsin it is interesting that cleavage occurs at this particular site before other susceptible bonds. This may indicate that the adjacent residues to the Leu10-Val11 bond are enhancing cleavage at this site due to the extended binding pocket of the enzyme (Powers *et al.*, 1977; Kay & Dunn, 1992). Alternatively, pepsin cleavage at other sites such as Gln15-Phe16 may relax the structure of the α-helical region which increases the enzymes accessibility to this bond and therefore increases the susceptibility of the site to proteolysis.

The cleavage sites I identified in the IGF-I moiety of long-R^3-IGF-I were identical to four of the six 'major' cleavage sites determined by Forsberg *et al.* (1990) as shown in Figure 1.6. The additional two 'major' pepsin cleavage sites found in their study were Asp53-Leu54 and Gly42-Ile43. These six sites were analogous with 'major' cleavage sites identified in IGF-II by Smith *et al.* (1989). Although pepsin digestion conditions were similar between my study and that of Forsberg *et al.* (1990) where an enzyme to substrate ratio of 1 : 20 was used in 10 mM HCl, Forsberg *et al.* used an incubation time of 3 to 4 h. This was presumably a more complete pepsin digestion of IGF-I than the 15 to 120 min time-points I

analysed and hence the lack of detection of these additional cleavage sites in my study is not surprising.

The results showed that the initial cleavage in the *N*-terminal extension peptide of long-R^3-IGF-I was not crucial for retention of biological activity. The resultant peptide, FVN-R^3-IGF-I, retained receptor binding affinity and the ability to stimulate protein synthesis activity. In fact, FVN-R^3-IGF-I had a greater affinity for the type 1 IGF-receptor than the parent material, long-R^3-IGF-I. The increased receptor affinity of the FVN-R^3-IGF-I peptide may be due to the removal of the extension peptide which could be acting as a physical impediment to receptor binding. The peptide fragments D and E (Figure 2.4) which were formed by pepsin cleavage at the other four identified sites, did not retain receptor affinity. As the presence of these fragments occurred early in the time-course of long-R^3-IGF-I degradation (15 to 30 min at an enzyme to substrate ratio of 1 : 100, Figure 2.1) it would appear that stabilisation of the cleavage sites forming these fragments is most crucial for retention of the peptides biological activity. Consequently, it was reasoned that bio-activity of an intra-gastric IGF-I molecule would be limited by pepsin cleavage at any of these sites, and hence all four cleavage points would be investigated by mutagenesis. The first cleavage site in the *N*-terminal extension peptide was not examined further since cleavage at this position resulted in the formation of a product with equal or greater bio-activities than long-R^3-IGF-I.

2.5.2 Basis for the design of the pepsin-resistant long-R^3-IGF-I analogues

2.5.2.1 General considerations

Having identified the key pepsin cleavage sites in long-R^3-IGF-I, a strategy was then developed for selecting single mutations that would be likely to stabilise the analogue against pepsin digestion while also retaining bio-activity. Mutations were designed based on two major considerations. These were: (a) the preference pepsin has for different cleavage sites and (b) the use of amino acid substitutions least likely to disturb protein structure and hence biological activity.

As noted earlier (Section 2.5.1), hydrophobic residues especially amino acids containing an aromatic ring in the side-chain are particularly favourable to pepsin digestion

(Fruton 1970). Therefore, predictions can be made whether amino acid changes at the cleavage sites will be effective based on the amino acid and side-chain specificity preferences shown by pepsin. To prevent loss of substantial biological activity, the mutations would also be selected to minimise changes in overall protein conformation of the analogue. The three-dimensional structure of the IGF-I molecule has been implicated in the biological activity of the peptide as shown by the existence of an IGF-I variant with mis-matched disulphide bonds that has markedly reduced biological activity (Hodgkinson *et al.*, 1989; Forsberg *et al.*, 1990). Minimal interference with the type 1 receptor-binding region was also considered important for retention of bio-activity, including for example, key tyrosine residues at positions 24 and 31 (Bayne et al., 1990a), plus areas of sequence conservation between functionally related molecules, IGF-I, IGF-II and insulin (Figure 1.1 and Figure 1.4). Together these considerations eliminated some putative pepsin-stable mutations. The pepsin cleavage site at the Tyr24-Phe25 bond was an example of this situation. As the Tyr24 residue has been shown to be directly involved in the binding of the IGF-I molecule to the type 1 receptor (Cascieri *et al.*, 1988; Maly & Luthi, 1988; Bayne *et al.*, 1990a) this residue was not mutated. Rather, Phe25 was selected for amino acid substitution, while realising that the mutation may show residual susceptibility to pepsin due to the retention of an aromatic amino residue at position 24 (Trout & Fruton, 1969).

The use of amino acid substitutions least likely to disturb protein structure were based on the work by Bordo & Argos (1991). In this theoretical study, Bordo & Argos calculated residue exchange matrices which give suggestions for 'safe' residue substitutions in site-directed mutagenesis. 'Safe' substitutions are those resulting in minimal disturbances of the protein fold and structural characteristics, locally as well as globally. The matrices represent point mutational preferences as observed in nine molecular families which included cytochromes c, haemoglobins, immunoglobins and various proteases. Equivalent residue pairs, either buried in the protein core or exposed at the protein surface, that had mutated and maintained similar unmutated environments were found in the various topological families. Residue exchange statistics and their significance were then determined by these authors. With this data, exchange matrices (Appendix I) of preferred and avoided amino acid

substitutions were deduced for residues in exposed, buried or either situation. Also included in the matrices, are the number of actual exchange counts observed.

The analogues were constructed as single-point mutations so that each modification could be characterised separately. A site not identified under my cleavage conditions but determined by Forsberg *et al.* (1990) as a major pepsin cleavage site [*viz.* Asp53-Leu54] was also used in this mutational study.

The next section outlines in detail the rationale behind the design of each analogue. Figure 2.7 is a summary showing the pepsin cleavage sites in long-R^3-IGF-I and the single-point mutations selected.

2.5.2.2 The Leu10-Val11 bond

Of the four initial pepsin cleavage sites observed in the IGF-I molecule, the Leu10-Val11 bond was one of the later cleavage sites. Both the amino acids, Leu10 and Val 11, are conserved throughout IGF-I, IGF-II and insulin. They are situated in an area of defined structure, the first α-helical domain of the molecule (Cooke *et al.*, 1991; Sato *et al.*, 1992). Cooke *et al.* (1991) from their structural determination report the side-chain of Leu10 is mostly buried while that of Val11 is exposed on the surface of the IGF-I molecule. Although the amino acid side-chain of Leu10 may be buried, the Leu10-Val11 peptide bond must be accessible for pepsin cleavage to occur at this site. Substitutions at either of these amino acids have not been described elsewhere, although the first 16 amino acid residues of the *B*-domain of IGF-I appear to be more critical for binding all the IGFBPs than to receptors (Bayne *et al.*, 1988; Oh *et al.*, 1993). Consequently, a conservative substitution at either Leu10 or Val11 which retains the helical structure was predicted to have a limited effect on type 1 receptor binding.

Pepsin is known to hydrolyse bonds containing aliphatic residues such as Leu and Val. However, Fruton (1974) reported that substitution of a Val or Ile at the P_1 position inhibits pepsin action. Furthermore, a substitution trend found by Bordo & Argos (1991) was that Leu residues are interchangeable with either Val or Ile, regardless of whether the amino acid was in an exposed or buried position, with more evidence of Val substitutions in cases of

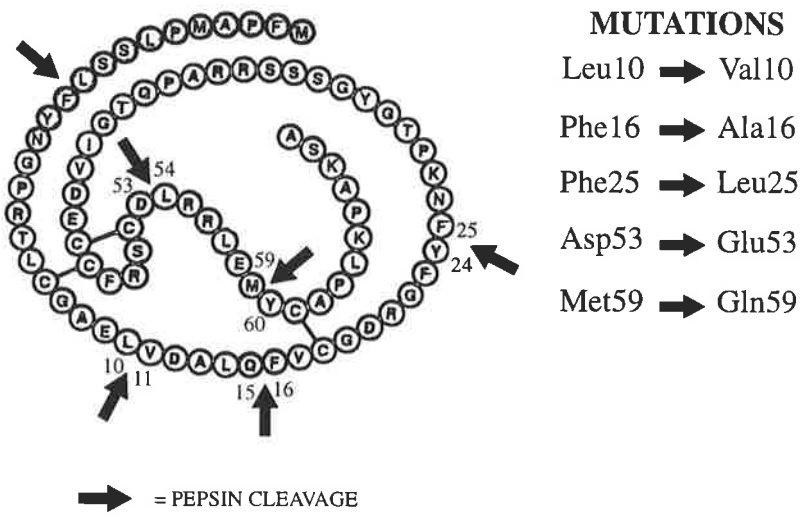

FIGURE 2.7
Pepsin cleavage sites in long-R^3-IGF-I and the selected point mutations.

70 % environmental similarity. Consequently, I decided to substitute Val for Leu at position 10. As an abbreviated terminology, this is described subsequently in the thesis as a 'Leu10Val' mutation, with equivalent terminology applied to the other mutations.

2.5.2.3 The Gln15-Phe16 bond

The Gln15-Phe16 bond was the first pepsin cleavage site observed in the IGF-I moiety. These amino acids are in a region of significant structural similarity between the IGFs and are situated at the end of the first α-helix in the *B*-domain of IGF-I. Single site mutations at either of these residues have not been reported, although a Tyr15-Leu16 IGF-I analogue based on the analogous insulin residues (Bayne *et al.*, 1988) has reduced IGF binding protein affinity and increased insulin and type 2 IGF-receptor binding. As the Phe16 residue is involved in IGFBP binding, it is likely to represent an exposed amino acid residue. This is supported by NMR studies which indicate that Phe16 is situated at the end of the first helix with the aromatic side chain covering the core of the peptide between the first and third helices (Cooke *et al.*, 1991).

As bonds containing aromatic amino acids are particularly favourable for hydrolysis by pepsin (Trout & Fruton, 1969), the Phe residue was selected for mutation rather than the Gln moiety of the bond. Bordo & Argos (1991) observed that Phe and Tyr are interchangeable with high statistical significance. However, as Tyr is an aromatic amino acid it would also be susceptible to pepsin cleavage. When the Phe residue is an exposed position in a protein, Tyr was the only statistically favoured substitution observed by Bordo & Argos (1991). Studies involving receptors (Bass *et al.*, 1991; Cunningham & Wells, 1989) and enzymes (Ellerby *et al.*, 1990; Carter & Wells, 1988) have shown that a general amino acid substitution to Ala can have a limited effect on protein structure. As Ala is a small abundant non-polar amino acid, it was chosen by these researchers to minimise unfavourable steric contacts and to avoid imposing new charge interactions or hydrogen bonds from the substituted side chains. Consequently a Phe16Ala mutation was selected for the Gln15-Phe16 bond.

2.5.2.4 The Tyr24-Phe25 bond

The second pepsin cleavage site observed in the IGF-I molecule was the Tyr24-Phe25 bond. These amino acids are exposed residues in a cleft on one face of the IGF-I molecule with their aromatic side-chains lying flat across the surface (Cooke *et al.*, 1990). The residues, Phe23, Tyr24 and Phe25 in IGF-I, are recognised as forming an important domain for the type 1 IGF-receptor binding (Cascieri *et al.*, 1988). Of these, Tyr24 has been directly implicated in receptor binding (Cascieri *et al.*, 1988; Maly & Luthi, 1988; Bayne *et al.*, 1990a) and was therefore not considered for mutation. Instead, the Phe25 amino acid was selected for mutation. In addition, since pepsin preferentially cleaves bonds with Phe residues at P'_1 (Fruton, 1970), this suggested a mutation at Phe25 rather than Tyr24 may limit pepsin proteolysis at this site. However, if cleavage is restricted at this peptide bond, it was recognised that pepsin proteolysis may increase at the adjacent minor cleavage site (Ph23-Tyr24) since Forsberg *et al.* did not observe cleavage at both these sites in one molecule (personal communication). Since substitution at the Phe25 amino acid would most likely affect receptor binding a conservative mutation was proposed. Using the matrices determined by Bordo and Argos, (1991) a Phe25Leu mutation is a favourable substitution regardless of the exposed or buried status of the Phe residue.

Although pepsin hydrolysis was not expected to be completely inhibited with a Phe25Leu mutation, due to the presence of the Tyr in the P_1 position (Dixon & Webb, 1964), partial stabilisation was anticipated (Fruton, 1974). Accordingly a Phe25Leu mutation was selected for the Tyr24-Phe25 bond.

2.5.2.5 The Met59-Tyr60 bond

Tyr60 is a highly conserved amino acid in IGF-I, IGF-II and insulin. This residue is likely to be involved in type 1 IGF-receptor binding as shown by its protection from iodination in the receptor-IGF-I complex (Maly & Luthi, 1988) and by the observation of a 20-fold loss in receptor affinity in the [Leu^{60}]IGF-I mutant (Bayne *et al.*, 1990a). Therefore, in an attempt to retain biological activity the Met59 amino acid was substituted rather than Tyr60.

At the Met59 position of IGF-I, there is published data of two single site mutations both with slightly decreased type 1 IGF-receptor binding (Peters *et al.*, 1985; Forsberg *et al.*, 1990). Mutation of Met59 to Thr59, the analogous residue in IGF-II, or to Norleu59 results in IGF peptides with 80 % and 70 % respectively the receptor affinity of IGF-I (Peters *et al.*, 1985; Forsberg *et al.*, 1990). In addition, a methionine sulphoxide form of IGF-I has been shown to have essentially retained receptor affinity as well as decreased pepsin cleavage between residues 59 and 60 compared to native IGF-I (Forsberg *et al.*, 1990). Together these studies further suggested the Met59 residue for mutation since substitution at this residue was not anticipated to markedly affect type 1 receptor binding. In addition, mutation at Met59 may have a limited effect on the structural conformation of the IGF molecule as the side-chain of Met59 is exposed (Forsberg *et al.*, 1990) which may allow accommodation of other substituted C_β residues*.

Using the exchange matrices (Bordo & Argos, 1991), Thr and Gln are the more frequent substitutions for an exposed Met residue although neither are statistically favoured. Bordo & Argos also report Gln as a frequent but not statistically significant substitution for Thr. As the [Thr^{59}]IGF-I analogue had an approximate equivalent biological activity to IGF-I (Peters *et al.*, 1985), I subsequently decided on a Gln59Met mutation at the Met59-Tyr60 bond. This mutation may also limit pepsin proteolysis at this site, since the presence of Met residues in tyrosine-containing peptides has been reported to increase susceptibility to pepsin cleavage (Dixon & Webb, 1964).

2.5.2.6 The Asp53-Leu54 bond

The amino acid residues, Asp53 and Leu54, in IGF-I are also conserved at the analogous residues in IGF-II. No single site mutations have been reported at either of these residues in IGF-I or IGF-II. In IGF-I, Asp53 is at the end of a loop structure which is constrained by the disulphide bond of Cys47-Cys52 connecting helices II and III, while Leu54 is the first residue in helix III (Sato *et al.*, 1993). In addition, Asp53 lies on the surface

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* The first residue in the side-chain of an amino acid, excluding glycine, is a carbon atom and this is labelled a β-carbon atom (C_β).

of the molecule between the patches of residues Ala8, Glu9, Asp12 and Gly1, Pro2, Phe49, Arg50, Arg55 and Arg56 (Cooke *et al.*, 1991) which may be implicated in interactions with the type 2 IGF-receptor (Bayne *et al.*, 1988; Cascieri *et al.*, 1989).

As Asp53 is an exposed amino acid and thereby accessible for pepsin cleavage, this was the residue chosen for mutation. Bordo and Argos (1991) report replacement of an exposed Asp amino acid by Glu as a statistically preferred substitution. In addition, both residues are negatively charged amino acids. Dixon & Webb, (1964) reported that glutamyl peptides have only been found to be hydrolysed by pepsin when they contain an aromatic amino acid. Consequently, as no aromatic amino acid is present at either residue 52 or 54, pepsin cleavage may be prevented at the Leu residue due to the presence of the Asp53Glu mutation. Hence the Asp53Glu substitution was selected as the mutation for the Asp53-Leu54 bond.

2.5.3 Summary

Five initial cleavage sites were found in long-R^3-IGF-I. The first proteolytic cleavage in long-R^3-IGF-I occured at the Leu10-Phe11 bond in the *N*-terminal extension peptide. As cleavage at this site resulted in a peptide with bio-activity equivalent to the parent molecule, mutagenesis was not investigated at this site. At the four other pepsin cleavage sites, the following mutations were proposed: Leu10Val, Phe16Ala, Phe25Leu and Met59Gln. An additional mutation, Asp53Glu, was also selected.

CHAPTER THREE
PRODUCTION AND CHARACTERISATION OF LONG-R^3-IGF-I ANALOGUES

3.1 INTRODUCTION

The study in Chapter 2 determined the kinetics of long-R^3-IGF-I pepsin digestion. Of the five initial pepsin cleavage sites identified in long-R^3-IGF-I, the four susceptible bonds in the IGF-I moiety were selected for mutagenesis. The sites of pepsin cleavage were the Leu10-Val11, Gln15-Phe16, Tyr24-Phe25 and Met59-Tyr60 bonds. The mutations selected at these sites, based on pepsin preference of cleavage as well as minimal disturbance of protein structure, were Leu10Val, Phe16Ala, Phe25Leu and Met59Gln. An additional mutation, Asp53Glu, was also designed for the Asp53-Leu54 cleavage site identified by Forsberg *et al.* (1990).

In this chapter, I will discuss the production of constructs encoding the analogues which were made as single point mutations using site-directed mutagenesis. These constructs were transformed into *Escherichia coli* where the peptides were expressed intracellularly as inclusion bodies using the expression system developed for the expression of IGF-I (King *et al.*, 1992; Francis *et al.*, 1992; see section 1.2.9.4). The long-R^3-IGF-I analogues were purified and the impacts of the mutations on pepsin resistance, type 1 IGF-receptor binding, IGFBP binding and protein synthesis stimulation were measured.

3.2 MATERIALS

3.2.1 General chemicals and reagents

Activated charcoal, agarose, bovine serum albumin (BSA: radioimmunoassay grade), bromophenol blue, dithioerythritol (DTE), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) and xylene cyanol were purchased from BDH Chemicals, Merck Pty. Ltd., Kilsyth, VIC, Australia. Bis-

acrylamide was obtained from BioRad Laboratories Inc., Herates, CA, U.S.A. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG, X-GAL) was obtained from Progen Industries Ltd., Darra, Qld., Australia. Ethylenediaminetetraacetic acid (EDTA) was purchased from Boehringer Mannheim Australia, Sydney, NSW, Australia. 2-hydroxyethyl disulphide was from Aldrich, Milwaukee, WIS, U.S.A. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Diagnostic Chemicals Ltd., Charlottown, Canada. Phenol (special grade) was from Wako Pure Chemical Industries Ltd., Osaka, Japan. All other reagents were of analytical grade.

The peptides, recombinant IGF-I and long-R^3-IGF-I (receptor grade), were provided by GroPep Pty. Ltd., Adelaide, SA, Australia. Porcine pepsin (EC 3.4.23.1) was purchased from Boehringer Mannheim Australia, Sydney, NSW, Australia. Whatman 3MM paper was obtained from Whatman International Ltd., Maidstone, England and X-ray film was from Konica Corporation, Tokyo, Japan.

3.2.2 Molecular biology reagents

Restriction enzymes were purchased from Pharmacia Biotech, Sydney, NSW, Australia and calf intestinal alkaline phosphatase (CIP) was from Boehringer Mannheim Australia, Sydney, NSW, Australia. The Altered Sites™ *in vitro* mutagenesis system was from Promega Corporation, Sydney, NSW, Australia and the GENECLEAN II kit was purchased from Bio101 Inc., La Jolla, CA, U.S.A. Deoxynucleotide triphosphate and dideoxynucleotide triphosphate mixes (dNTP and ddNTP, respectively) were obtained from Biotech International, Bentley, WA, Australia and Sequenase® Version 2.0 was obtained from United States Biochemical, Cleveland, OH, U.S.A. Adenosine 5'-triphosphate ribonucleoside (rATP), ampicillin, dithiothreitol (DTT: molecular biology reagent), DNase-free RNase A, spermidine (N-[3-aminopropyl]-1,4-butanediamine), tetracycline and glass beads (212 to 300 μm) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. All other molecular biology materials, including synthetic oligonucleotides, were from Bresatec Ltd, Adelaide, SA, Australia.

3.2.3 *Escherichia coli* strains

DH5α	*sup*E44, Δ*lac*U169, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, λ^-, *rel*A1, [ϕ80*lac*ZΔM15]
JM109	*thi*, *sup*E44, Δ(*lac-pro*AB), *end*A1, *hsd*R17 (rk^-, mk^+), *rec*A1, *gyr*A96, λ^-, *rel*A1, [F', *tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$]
JM101	*sup*E, *thi*, Δ(*lac-pro*AB) [F', *tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$]
BHM 71-18 mut S	*thi*, *sup*E, Δ(*lac-pro*AB), [*mut*S::Tn10], [F', *pro*A^+B^+, *lac*$I^qZ\Delta M15$]

3.2.4 Bacterial culture media

All bacterial media and plates were prepared by Mrs. J. Brinkman, Biochemistry Dept., University of Adelaide, Adelaide, SA, Australia. Luria-Bertain (LB) media contains 1 % (v/v) bactotryptone, 0.5 % (w/v) bactoyeast extract, 0.17 M NaCl, pH 7.0. Minimal media (Min A) contains 50 mM Na_2HPO_4, 22 mM KH_2PO_4, 8 mM NaCl, 20 mM NH_4Cl, 0.4 % (w/v) glucose. Media plates were prepared by the inclusion of 1.2 % (w/v) agar. Bactotryptone, bactoyeast extract and agar were purchased from DIFCO Laboratories, MI, U.S.A.

3.2.4 Cell culture materials

Cell culture materials were purchased as described in Section 2.2 with routine maintenance and subculturing of cell lines carried out by Miss. S. Tilley (CRC for Tissue Growth and Repair, Adelaide, SA, Australia).

3.2.5 Chromatography materials

High performance liquid chromatography (HPLC) equipment was from Millipore-Waters (Sydney, NSW, Australia). The radial compression Novapak C4 column was also obtained from Millipore-Waters and the reverse-phase microbore C4 column was purchased from Brownlee Laboratories (Santa Clara, California, U.S.A). The Fast Protein Liquid Chromatography (FPLC) equipment and resins, Sephadex G-25M, S-Sepharose Fast Flow,

Sepharose CL-6B were purchased from Pharmacia Biotech Sydney, NSW, Australia. The C18 Matrex silica was obtained from Amicon, Danvers, MA, U.S.A.

HPLC grade acetonitrile (HiPerSolv™) was purchased from BDH Chemicals Ltd., Kilsyth, VIC, Australia and trifluoroacetic acid (TFA) was from Fluka Chemie, Buchs, Switzerland. Milli-Q water was obtained using Milli-Q filtering apparatus (Millipore-Waters, Sydney, NSW, Australia) and 0.22 μm GV filters were also purchased from Millipore-Waters.

3.2.6 Radiochemicals

Carrier-free $Na^{125}I$ and [4,5-^{3}H]leucine were obtained from Amersham Australia Pty. Ltd., Sydney, NSW, Australia. [α-^{32}P]dATP (3 000 Ci/mmol) were purchased from Bresatec Ltd., Adelaide, SA, Australia.

3.3 METHODS

All techniques were performed by myself unless otherwise stated.

3.3.1 Standard molecular biology methods

Generally, the molecular biology methods used were as described in Sambrook *et al.* (1989) unless otherwise mentioned.

3.3.1.1 Growth and transformation of *E. coli*

The *E coli* strains, JM109, JM101 and BHM 71-18 mut S were routinely maintained on Min A medium with the appropriate antibiotic, when transformed with a plasmid. The *E. coli* strain DH5α was maintained on LB media.

Glycerol stocks of the *E. coli* strains were aseptically streaked out onto LB agar plates and grown overnight at 37° C. A single colony was then inoculated into 2 ml of LB, except for strain BHM 71-18 mut S, which was grown in Min A containing 12.5 μg/ml tetracycline. Following overnight culture at 37° C with shaking, the strains were subcultured at a 1/100 dilution into fresh LB and cultured with shaking at 37° C for approximately 2 h

until the absorbance at 600 nm was between 0.3 to 0.6. The cells were pelleted by centrifugation for 5 min at 3 000 g then resuspended in 2.5 ml 50 mM $CaCl_2$, 20 mM $MgCl_2$ and left on ice to become competent for at least 60 min. Competent cells (200 µl) were incubated on ice with 5 µl of ligation reaction or other circular DNA for at least 40 min. Cells were heat shocked at 42° C for 2 min, left on ice for 2 min, then 100 µl of LB with 20 mM glucose was added. Cells were incubated at 37° C for 30 min, plated onto an appropriate plate containing antibiotic and incubated overnight at 37° C.

3.3.1.2 Preparation of plasmid DNA

Alkaline-lysis method : A single colony were used to inoculate 2 mls of LB containing 50 µg/ml ampicillin which was then grown overnight at 37° C with shaking. A 1.5 ml aliquot of the overnight culture was centrifuged for 5 min at 2 000 g, the supernatant was aspirated and the cell pellet suspended in 90 µl of cold TES (25 mM Tris, pH 8.0, 10 mM EDTA, 15 % (w/v) sucrose). Freshly prepared 0.2 M NaOH, 1 % (w/v) SDS (180 µl) was added to the pellet, which was mixed by gentle inversion, then incubated at room temperature for 2 min. An aliquot of 3 M sodium acetate, pH 4.6 (135 µl) was added, the pellet was mixed again by gentle inversion then centrifuged at 10 000 g for 20 min at room temperature. The supernatant was digested with 2 µl of DNase-free RNase A (10 mg/ml) at 37° C for 20 min, then extracted with an equal volume of Tris, pH 8.0, saturated phenol : chloroform mix (200 µl of each). The sample was vortexed and centrifuged at 10 000 g for 5 min at room temperature before approximately 400 µl of the aqueous supernatant was added to 1 ml of cold 100 % ethanol and precipitated for 15 min at room temperature. Precipitated DNA was centrifuged at 10 000 g for 20 min at room temperature, washed with 500 µl of 70 % (v/v) cold ethanol, then resuspended in 40 µl of 0.1 mM EDTA, pH 8.0. A 1 µl sample was run on a 1 % agarose mini-gel electrophoresis to estimate DNA quantity and purity.

Boiling method : DNA purified by this procedure was used as a template for the mutagenesis, as well as for sequencing potential mutants and checking transformations. A 1.5 ml aliquot of an overnight culture was centrifuged as described above. The cell pellet was gently loosened, resuspended in 200 µl of STET (0.1 M NaCl, 10 mM Tris, pH 8.0, 1 mM

EDTA, 5 % (w/v) Triton X-100) then digested with 10 µl of lysozyme (12.5 mg/ml in 10 mM Tris, pH 8.0) at 100° C for 2 min. Cells were centrifuged at 10 000 g for 15 min at 4° C and the pellet removed with a toothpick. To ensure no cell debris remained, the supernatant was recentrifuged for a further 5 min. An aliquot of supernatant (200 µl) was added to 200 µl of isopropanol and precipitated for 5 min on ice. Precipitated DNA was centrifuged at 10 000 g for 10 min at room temperature, the pellet washed twice with 1 ml 70 % (v/v) cold ethanol then resuspended in 20 µl of 0.1 mM EDTA, pH 8.0. DNA quantity and purity was assessed by agarose mini-gel electrophoresis.

3.3.1.3 DNA purification

GENECLEAN isolation of DNA : DNA fragments larger than approximately 400 bp were purified using the GENECLEAN kit, according to the manufacturer's instructions. Briefly, the DNA was isolated by electrophoresis on an agarose TBE (50 mM Tris, pH 8.0, 40 mM boric acid, 0.1 mM EDTA) gel, visualised by staining with ethidium bromide, then excised under long wave ultra violet (UV) light. The gel slice was solubilised by heating at 55° C for 5 min with 500 µl of NaI solution and 50 µl of TBE modifier. Binding of the DNA to 5 µl of glassmilk solution proceeded for 5 min at room temperature. The glassmilk-DNA complex was pelleted by centrifugation for 5 sec at 10 000 g then washed three times in 'New Wash' solution (200 µl), with gentle resuspension and pelleting between washes. The DNA was eluted from the glassmilk beads by resuspending the pellet in 10 µl of water then incubating at 60° C for 10 min. The glassmilk beads were repelleted by centrifuging for 15 sec at 10 000 g, and the supernatant retained. The elution was repeated and supernatants pooled. DNA recovery was assessed by 1 % agarose mini-gel electrophoresis.

Electroelution isolation of DNA : Small DNA fragments, less than 400 bp, were purified using electroelution. The DNA was isolated by electrophoresis on a 2 % agarose TBE gel, visualised by staining with ethidium bromide, then excised under long wave UV light. The gel slice was electrophoresed in dialysis tubing, presoaked by boiling in 1 mM EDTA for approximately 2 min, with 400 µl of 10 mM Tris, pH 7.5, 0.1 mM EDTA for 60 min at 75 mA. The DNA fragment was then isolated by phenol : chloroform extraction

and ethanol precipitation, as described for mutagenic reactions prior to electroelution (Section 3.3.1.6). DNA recovery was assessed by 2 % agarose mini-gel electrophoresis.

Sepharose CL-6B spin column DNA purification : Sepharose CL-6B spin columns were used to desalt and purify DNA prior to sequencing and mutagenesis. A mini-column was made by placing a drop of acid-washed glass beads in the base of a 0.5 ml Eppendorf tube, which had been previously pierced with a 21 gauge needle, then overlaying 500 µl of Sepharose CL-6B equilibrated in 10 mM Tris, pH 7.5, 0.1 mM EDTA. The column was spun at approximately 500 g for 3 min at room temperature and the eluate was discarded. The mini-column Eppendorf tube was then placed inside an intact 1.5 ml Eppendorf tube, a DNA sample (50 µl) was loaded onto the dry Sepharose CL-6B column bed, and the column was recentrifuged as above. The eluate, equivalent to the load volume, was retained in the 1.5 ml Eppendorf tube and contained DNA free of small nucleic acids, proteins and salts. DNA recovery was then assessed by 1 % agarose mini-gel electrophoresis.

3.3.1.4 Dephosphorylation of vector DNA

The linearised and purified vector was dephosphorylated with CIP. The vector was incubated in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$ with 2 U of CIP for 15 min at 37° C followed by 15 min at 65° C. A further 2 U of CIP was added and reincubated as above. The CIP was then removed by phenol : chloroform extraction and ethanol precipitation before ligation, as described in Section 3.3.1.6.

3.3.1.5 Ligation of DNA

A ligation mix (total volume of 10 µl) was set up with a 2-3 molar excess of DNA insert over linearised vector (dephosphorylated) in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM rATP with 2U of T4 DNA ligase. At the same time a negative control (vector alone without DNA insert) was also set up. Ligations occurred for 2-4 h at room temperature or 18 h at 14° C. DNA was then transformed into competent *E. coli* as described in Section 3.3.1.1.

3.3.1.6 Electroporation

Electroporation was used when transforming mutagenic reactions into BMH 71-18 mut S. Competent cells for electroporation were prepared by R. King, Department of Biochemistry, University of Adelaide, Adelaide, SA, Australia. Briefly, a 5 ml overnight culture was used to inoculate 500 ml of LB, which was grown at 37° C until the absorbance at 600 nm was approximately 0.7. Cells were chilled on ice for 15 min then centrifuged at 2 000 g, 4° C for 15 min. Cells were washed by a series of resuspensions followed by centrifugation (2 000 g, 4° C, 15 min). The initial resuspension was to the same volume in cold water, the second to half the original volume in cold water, and the third to 1/25th the original volume in chilled 10 % (v/v) glycerol, and finally to 1/360th the original volume in chilled 10 % (v/v) glycerol. Aliquots (40 µl) were stored at -70° C.

Before electroporation, the mutagenic reactions (30 µl) were phenol:chloroform extracted by diluting the reactions to 100 µl then adding 50 µl of Tris, pH 8.0, saturated phenol and 50 µl of chloroform. The tubes were vortexed and centrifuged for 2 min at 10 000 g. The aqueous phase was then re-extracted with an equal volume of chloroform. Glycogen (20 µg) and 3 M sodium acetate, pH 5.2 (1/10th the volume) were added to the supernatant followed by 2.5 volumes of cold ethanol. The DNA was left to precipitate at -20° C for approximately 4 h before centrifuging at 10 000 g, 4° C for 30 min. The pellet was washed three times with 50 µl of 70 % (v/v) cold ethanol, air dried, then resuspended in 10 µl of water and left on ice.

An aliquot of competent cells, thawed on ice, was incubated for 30 min on ice with 2 µl of DNA, prepared as described above. Cells were transferred to a pre-cooled electroporation vessel and shocked at 25 µF, 2.0 kV, 200 Ω, time constant ~ 4.8. Cells were washed from the chamber with 1 ml SOC media (2 % (w/v) bactotryptone, 0.5 % (w/v) bactoyeast extract, 8 mM NaCl, 2.5 mM KCl, 20 mM glucose, pH 7.0) and incubated at 37° C for 20 min. The cells were then plated onto LB containing 100 µg/ml ampicillin, after briefly centrifuging at 4 000 g, removing approximately 900 ul of media then resuspending the pellet in the remaining media.

3.3.1.7 DNA sequencing

DNA sequencing was based on the dideoxy-sequencing method of Sanger, (1977) using the modified T7 DNA polymerase, Sequenase® Version 2.0, according to the manufacturer's protocol. Briefly, DNA was treated with 2 µl of 10 mg/ml DNase-free RNase A for 15 min at 37° C, denatured in 0.2 M NaOH, 0.2 mM EDTA at 37° C for 15 min then desalted on a Sepharose CL-6B spin column (Section 3.3.1.3). The denatured DNA (1-2 µg) was annealed with universal sequencing primer (10-50 ng) in 40 mM Tris, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl (total volume of 10 µl) at 65° C for 5 min, 37° C for 30 min then room temperature for a further 30 min. The annealed primer was labelled for 2-5 min at room temperature with 1 µl 0.1 M DTT, 2 µl labelling nucleotide mixture (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 5 µCi [α-^{32}P]dATP and 1 U of Sequenase® enzyme in a total volume of 15 µl. The labelling reactions were terminated by transferring 3.5 µl of the reaction to 2.5 µl of each of the four dideoxy nucleotide mixtures (80 µM dNTP, 50 mM NaCl, 8 µM ddNTP) and incubating at 37° C for 2-5 min. Tubes were then stored on ice after the addition of 4 µl stop solution (95 % (v/v) deionized formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue and 0.05 % (w/v) xylene cyanol). Sequencing reactions were denatured by heating at 95° C for 5 min then were loaded onto a 0.3 mm, 7 M Urea, TBE, 6 % polyacrylamide gel (w/v, 25 : 1, acrylamide : bis-acrylamide) and electrophoresed at 40 Watts constant power (approximately 30 mA, 1500 V) for the appropriate migration distance. The gel was fixed by soaking for 30 min in 10 % (v/v) acetic acid, 20 % (v/v) ethanol then was transferred to Whatman 3MM paper and dried under vacuum at 70° C for 60 min. The dried gel was exposed to X-ray film overnight at room temperature.

3.3.1.8 Purification and phosphorylation of oligomers

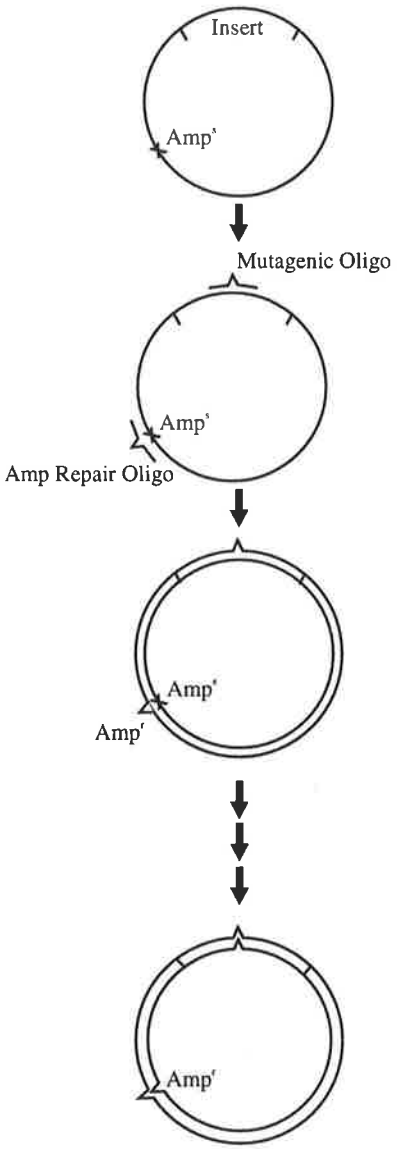
Oligomers (50 µg in 15 µl) were heat denatured in an equivalent volume of 95 % (v/v) deionized formamide and electrophoresed on a 0.5 mm, 7 M Urea, 50 mM Tris, pH 8.0, 0.1 mM EDTA, 40 mM boric acid, 18 % polyacrylamide gel (w/v, 25 : 1, acrylamide : bis-acrylamide) at 500 V. Oligomer bands were visualised and excised by shadowing under UV light onto cellulose/fluor 300 polyethyleneimine impregnated chromatography paper

(Machery-Nagel and Co., Germany). Oligomers were eluted into water (500 µl) overnight at 37° C then precipitated with two volumes of ethanol and 1/10th volume of 3 M sodium acetate, pH 5.2 at 4° C overnight. The pellet was washed with 70 % (v/v) ethanol, resuspended in 100 µl of water and stored at -20° C. Oligomers, approximately 200 pmoles, were 5' phosphorylated using 4.5 U of T4 polynucleotide kinase in 100 mM Tris, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM rATP, total volume 30 µl, for 45 min at 37° C. Phosphorylated oligomers were stored at -20° C after kinase activity was inactivated by heating at 65° C for 10 min.

3.3.2 Generation of DNA clones

The cDNA constructs encoding the long-R^3-IGF-I analogues were constructed by site-directed mutagenesis using a modification of the Altered Sites™ in vitro mutagenesis system. This system is based on the use of a second mutagenic oligonucleotide which confers antibiotic resistance on the mutant DNA strand (Figure 3.1). The cloning and mutagenesis procedures used in the construction of the plasmids are described in the following section. The vectors, including the final pSELECT™-1/long-R^3-IGF-I mutagenesis template, are illustrated in Figure 3.2.

The cDNA of long-R^3-IGF-I in pBluescriptSK+ (present as an *Eco* R1- *Hind* III insert) was subcloned into pSELECT™-1 (the template for mutagenesis) by the following method. DNA of the vectors, prepared by the alkaline-lysis method (Section 3.3.1.2), were digested with *Xba* I and *Kpn* I in 'Superduper buffer' (33 mM Tris acetate pH 7.8, 62.5 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, 0.5 mM DTE). Digestions, total volume 30 µl, were performed at 37°C for approximately 2 h. The pSELECT™-1 vector and the long-R^3-IGF-I DNA insert were isolated by GENECLEAN II (Section 3.3.1.3). The vector and insert were ligated (Section 3.3.1.5), transformed into competent *E. coli* DH5α (Section 3.3.1.1) and plated on LB + 10 µg/ml tetracycline plates with 500 µg of IPTG and 1.2 mg BCIG for subsequent blue/white selection. The substrates, IPTG and BCIP are metabolised to a blue precipitation in the presence of a functional β-galactosidase (*lac*Z) gene product. Transformants with DNA inserts have the plasmid encoded portion of



Recombinant dsDNA
template (Amp^s, Tet^r)

1. Anneal ampicillin repair oligo
   and mutagenic oligo.

2. Synthesize mutant strand with T4 DNA
   polymerase and ligase.

3. Transform BMH 71-18 mutS.
   Grow in media + ampicillin.
4. Prepare mini-prep DNA.
5. Transform JM109.
   Select mutants on ampicillin plates.
6. Screen for mutants by
   direct sequencing.

FIGURE 3.1
Schematic representation of the Altered Sites *in vitro* mutagenesis procedure.

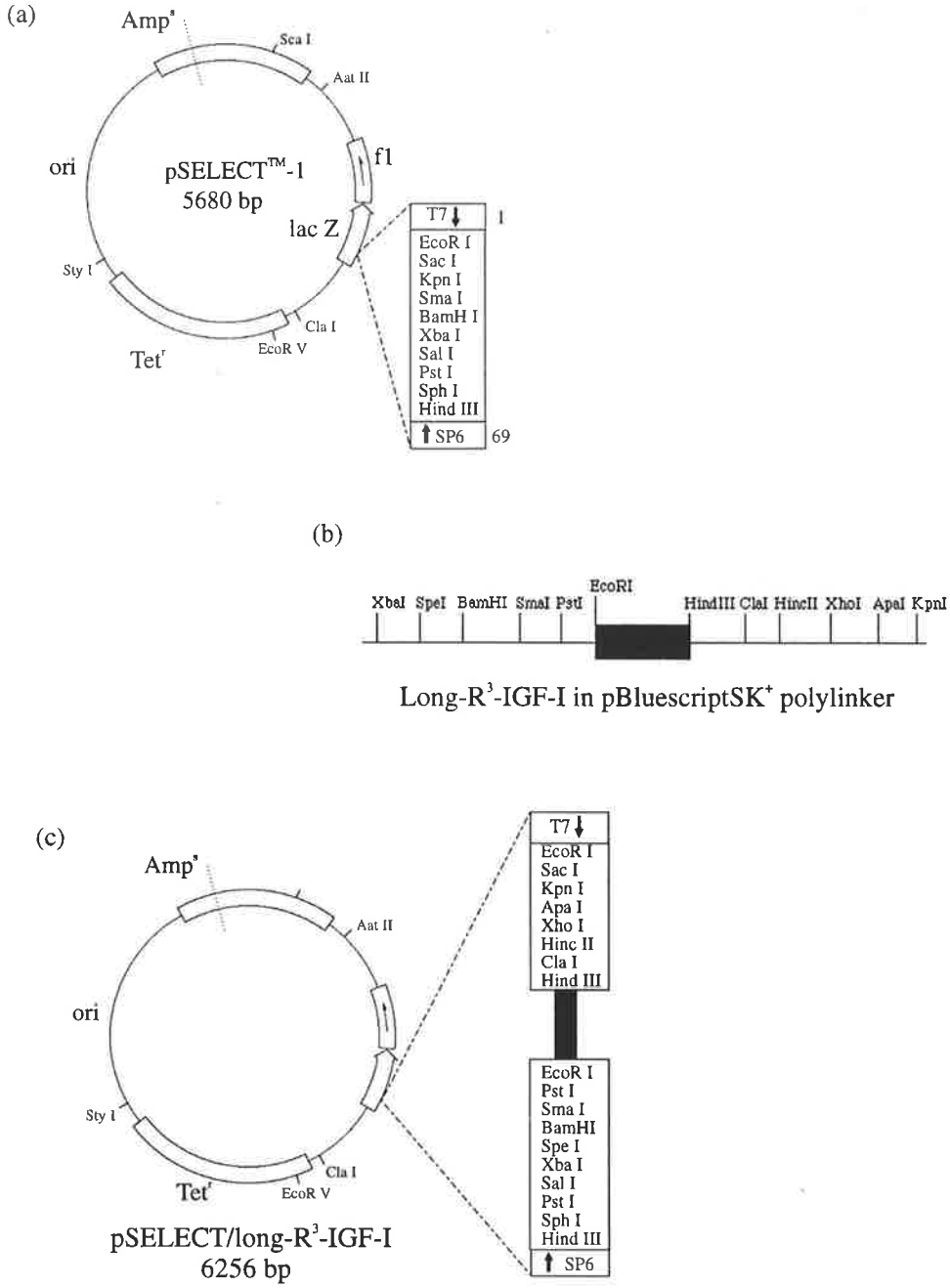


FIGURE 3.2
Construction of the template plasmid (pSELECT/long-R^3-IGF-I) used for mutagenesis.
The plasmid, pBluescriptSK$^+$ containing long-R^3-IGF-I inserted EcoR I/Hind III into the polylinker, was digested with *Xba* I and *Kpn* I and the long-R3-IGF-I fragment (indicated as a solid area) isolated. This fragment (b) was then ligated to *Xba I-* and *Kpn* I- cut pSELECT^{-1} (a) generating the template plasmid, pSELECT/long-R^3-IGF-I (c) used for mutagenesis.

the *lac*Z gene disrupted which results in loss of functional β-galactosidase activity and hence a white colony.

A double-stranded DNA preparation of the plasmid, pSELECT/long-R^3-IGF-I, was prepared by the boiling method (Section 3.3.1.2), then treated with DNase-free RNase A (20 μg, for 15 min at 37° C), denatured in 0.2 M NaOH, 0.2 mM EDTA at 37° C for 15 min and desalted on a Sepharose CL-6B spin column (Section 3.3.1.3). This DNA was used as the template for mutagenesis. The following synthetic oligonucleotides with 12 codons of complementary DNA flanking the region of mutation were used to generate the single point mutations: Phe16Ala (TTC to GCG), Leu10Val (CTG to GTG), Phe25Leu (TTC to CTG), Asp53Glu (GAC to GAA), Met59Gln (ATG to CAG). The mutation codons were optimised for usage in *E. coli* as in the parent long-R^3-IGF-I construct.

Oligo Ala^{16}, 5'-GTC ACC GCA AAC CGC CTG CAG AGC GTC-3';

Oligo Val^{10}, 5'-CAG AGC GTC AAC CAC TTC AGC ACC GCA-3';

Oligo Leu^{25}, 5'-GGT CGG TTT GTT CAG GTA GAA GCC ACG-3';

Oligo Glu^{53}, 5'-CAG ACG ACG CAG TTC GCA AGA ACG GAA-3';

Oligo Gln^{59}, 5'-CGG AGC GCA GTA CTG TTC CAG ACG ACG-3'.

The oligonucleotides, including the ampicillin repair oligonucleotide, were purified and phosphorylated as described in Section 3.3.1.8. Mutagenic annealing reactions containing mutagenic and repair oligomers at 1.25 pmoles and 0.25 pmoles respectively, were incubated with DNA template (approximately 100 ng) in 20 μl of 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl for 60 min at 37° C followed by 20 min on ice. Negative controls, no oligomers and repair oligomer only, were also included. Synthesis of the mutant strand was achieved by adding 6 μl of synthesis buffer (50 mM Tris, pH 7.5, 2.5 mM of each dNTP, 5 mM rATP, 20 mM DTT) with 2 U of T4 DNA ligase and 1 U of T4 DNA polymerase, giving a final volume of 30 μl. Tubes were incubated at 37° C for 120 min followed by an overnight incubation at 14° C after the addition of a further 2 U of ligase per tube. Reactions were then transformed into competent *E. coli* BMH 71-18 mut S by electroporation (Section 3.3.1.6). Twelve colonies from each mutagenic reaction were selected, the plasmid DNA was isolated (Section 3.3.1.2) then transformed into *E. coli* JM109 (Section 3.3.1.1) on LB + 100 μg/ml

ampicillin plates. Plasmid DNA, isolated by the boiling method (Section 3.3.1.2), was used to screen for successful mutagenesis by DNA sequencing (Section 3.3.1.7).

The long-R^3-IGF-I mutations were subcloned into the [Met^1]-pGH(1-11)-Val-Asn-[Arg^3]-IGF-I expression vector (Figure 3.3; Francis *et al.*, 1992) by the following method. Plasmid DNA of the expression vector and the long-R^3-IGF-I mutations were isolated by alkaline-lysis (Section 3.3.1.2). The DNA was digested with *Hpa* I and *Hind* III in 'Superduper buffer' (33 mM Tris acetate pH 7.8, 62.5 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, 0.5 mM DTE) at 37° C for approximately 2 h. The linearised expression vector was purified by GENECLEAN (Section 3.3.1.3) then dephosphorylated using CIP. The long-R^3-IGF-I analogue DNA inserts were isolated by electroelution (Section 3.3.1.3). The *Hpa* I-*Hind* III fragments, containing the required mutations, were then ligated into the expression vector and transformed into *E coli* JM101. The mutations and complete long-R^3-IGF-I codon sequence was checked by DNA sequencing (Section 3.3.1.7) prior to fermentation. Interestingly, in addition to the Phe25Leu mutation in the construct of long-R^3L^{25}-IGF-I, a nucleotide substitution was noted at Thr40. However, this was a silent mutation which also coded for the Thr amino acid; codon ACC had been replaced by ACT.

3.3.3 Induction of analogues

The expression from the long-R^3-IGF-I plasmids in *E coli* JM101 was tested in small cultures prior to fermentation. Overnight cultures were subcultured into 5 ml of Min A + 100 µg/ml ampicillin and grown for 2.5 h at 37° C. A 1 ml sample was taken as an uninduced control then 250 µM IPTG was added to the remaining culture which was grown for another 60 min before a 1 ml induced sample was taken. The cell samples were centrifuged for 5 min at 10 000 g and the induced and uninduced cell pellets were lysed respectively with 60 µl and 30 µl of lysis buffer (2 % (w/v) SDS, 10 % (v/v) β-mercaptoethanol). Proteins were analysed using 8-12 % gradient polyacrylamide gels on a "PhastSystem" (Pharmacia Biotech, Sydney, NSW, Australia) according to the

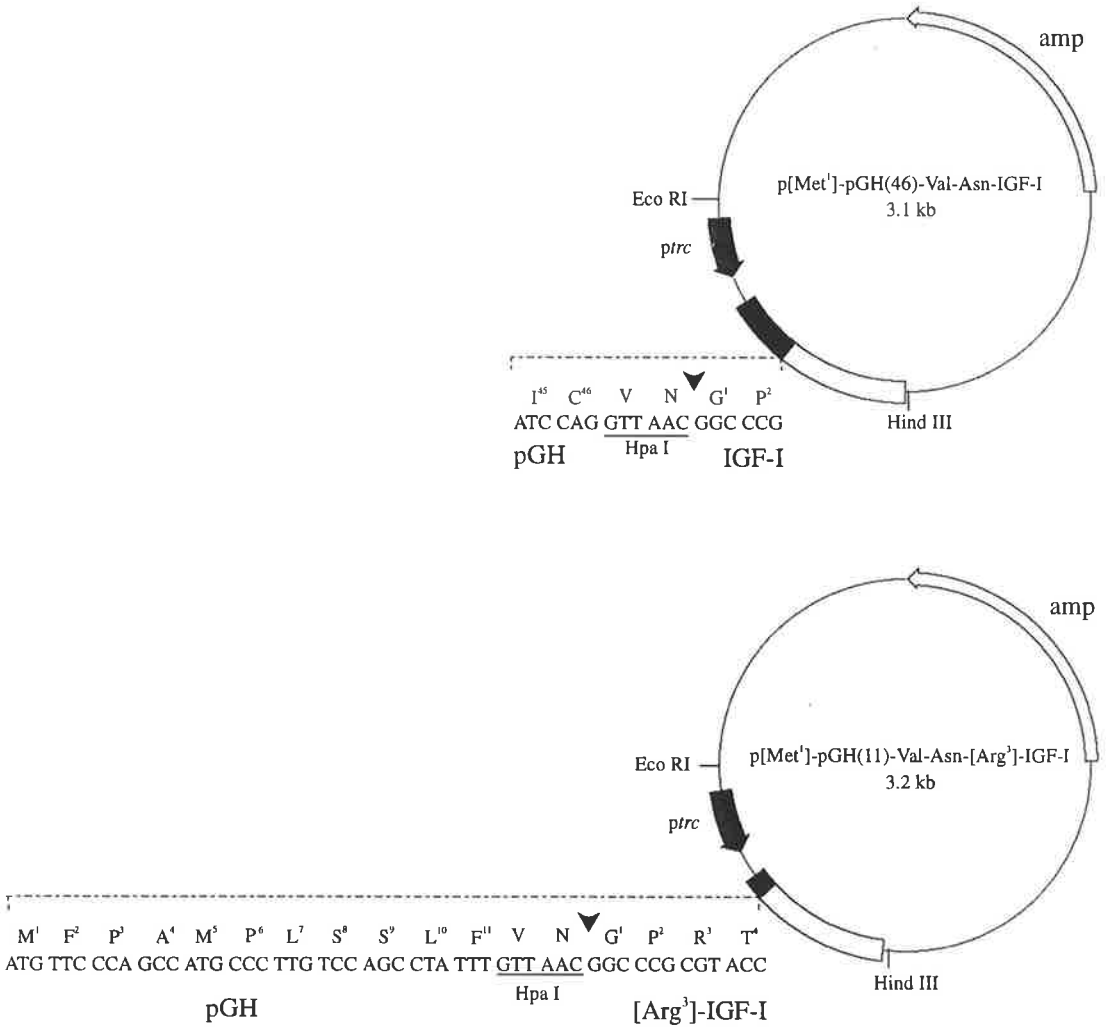


FIGURE 3.3
Schematic representation of the p[Met^1]-pGH(1-11)-Val-Asn-[Arg^3]-IGF-I expression vector and its derivative vector, p[Met^1]-pGH(1-46)-Val-Asn-IGF-I.
The solid areas represent p[Met^1]-pGH sequences. The IGF-I sequences are indicated by open areas. The junction DNA and encoded protein sequences are in the exploded view beside the figures; the numbering of amino acids proceeds from the *N*-terminus of [Met^1]- pGH and the arrow head represents a redundant hydroxylamine-labile Asn-Gly bond. The expression vectors also contain a trc promoter (p*trc*), an optimised ribosome binding site, a transcription termination sequence and a gene conferring ampicillin resistance (amp).

manufacturer's instructions. Samples were electrophoresed beside low molecular weight markers (Pharmacia Biotech, Sydney, NSW, Australia).

3.3.4 Fermentation and isolation of inclusion bodies

Fermentations were performed in 2 litre bioreactors (Applikon^R Autoclavable Bioreactor, Schiedam, Holland), essentially as described by King *et al.* (1992). Briefly, *E. coli* cells carrying the expression vector were grown in 200 mM glucose, 2.3 mM $MgSO_4$, 30 mM NH_4Cl, 6.9 mM K_2SO_4, 12 mM KH_2PO_4, 18 mM Na_2HPO_4, 0.3 mM $Na_3C_6H_5O_7$, 30 µM $MnSO_4$, 30 µM $ZnSO_4$, 3 µM $CuSO_4$, 72 µM $FeSO_4$ and 0.12 mM thiamine, pH 7.0 at 37° C until the absorbance at 600 nm was approximately 8.0. The cultures were induced with 250 µM IPTG, grown for a further 5 h then stored at 4° C. Fermentations were performed by Mrs. C. Senn, Bresatec Ltd., Adelaide, SA, Australia.

Figure 3.4 illustrates the purification sequence used for isolation of the long-R^3-IGF-I analogues.

Cells were disrupted by 5 passes through a homogeniser at 9 000 psi and then the inclusion bodies were isolated by centrifugation at 13 200 g for 15 min at 4° C. The inclusion body pellet was washed by suspension at 5 % (v/v) in 30 mM NaCl, 10 mM KH_2PO_4 buffer pH 7.8 and recentrifugation at 4 300 g for 15 min followed by a second resuspension and then recentrifugation for 10 min at 3 000 g. The washed inclusion bodies were then stored at -20° C prior to purification.

3.3.5 Refolding and purification of fusion proteins

The method used for refolding and purifying the fusion proteins was based on the procedures previously used for purifying human IGF-I and its analogues (King *et al.*, 1992; Francis *et al.*, 1992) and chicken IGF-I (Upton *et al.*, 1992). The refolding and purification steps were monitored throughout the purification procedure using analytical reverse phase HPLC as described by Upton *et al.* (1992). A linear gradient of 15 to 50 % (v/v) acetonitrile over 35 min in the presence of 0.1 % (v/v) TFA at 0.5 ml/min was used on a Brownlee

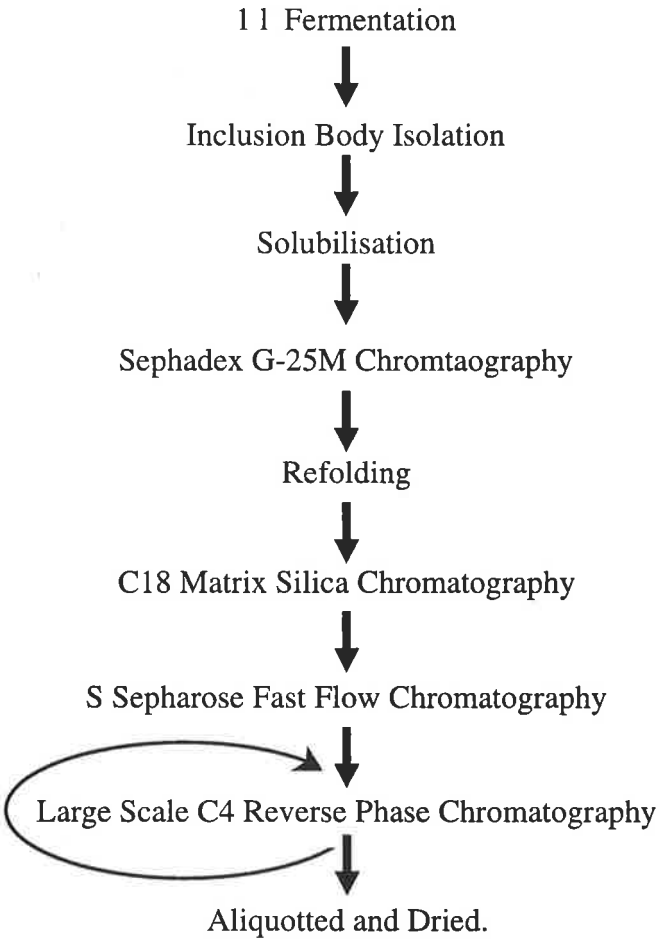


FIGURE 3.4
Purification sequence used for purification of the long-R^3-IGF-I analogues.

microbore C4 column (2.1 x 100 mm). Elution of protein was monitored by absorbance at 215 and 280 nm. Quantitation of the peptides was determined by converting the area under the absorbance profile to protein concentration using calculated extinction coefficients and Q values based on Buck *et al.* (1989), Table 3.1. All solvents and buffers were prepared in Milli-Q water and filtered through a 0.22 µm membrane prior to use.

Inclusion body pellets were solubilised in approximately 60 mls of 8 M urea, 40 mM glycine, 100 mM Tris, 0.5 mM $ZnCl_2$, 20 mM DTT, pH 9.1, centrifuged at 12 000 g for 15 min then desalted on a Sephadex G-25M column (5 x 20.5 cm) a flow rate of 10 ml/min. The Sephadex G-25M column was pre-equilibrated in the urea buffer, pH 9.1 with 1.6 mM DTT.

Desalted, reduced fusion proteins were refolded under oxidising conditions. The Sephadex G-25M eluate was diluted to a final protein concentration of less than 0.125 mg/ml in 2 M urea, 0.4 mM DTT with the diluent, 40 mM glycine, 100 mM Tris, 10 mM EDTA, pH 8.5 then 2-hydroxyethyl disulphide was added at a concentration of 60 µl per litre. The oxidation reaction occurred overnight with stirring at room temperature. On completion, the refolding reaction was stopped by lowering the pH to 2.1 using concentrated HCl.

The refolded peptides were concentrated on a C18 Matrex silica column (2.6 x 11.5 cm) equilibrated in 10 % (v/v) acetonitrile with 0.1 % (v/v) TFA. The peptides were loaded onto the column, washed with 10 % (v/v) acetonitrile with 0.1 % (v/v) TFA until baseline absorbance was reattained, then eluted using a linear gradient of 12 to 80 % acetonitrile with 0.1 % (v/v) TFA at 10 ml/min. The peptide fraction was then loaded onto an S-Sepharose Fast Flow column (2.6 x 11 cm) equilibrated in 1 M acetic acid. The column was washed with 50 mM ammonium acetate, pH 4.8, at 10 ml/min followed by a linear gradient over 30 min to 100 mM ammonium acetate, 2 M NaCl, pH 5.2. The peptides, collected as a single fraction, were further purified by two reverse phase HPLC steps using a radial compression Novapak C4 column. The first HPLC step used a linear gradient from 24 to 44 % (v/v) acetonitrile in 0.1 % (v/v) TFA over 200 min with a flow rate of 5 ml/min. Collected peptide fractions were diluted 2 fold in Milli-Q water containing 0.1 % (v/v) TFA, reloaded onto the column and re-eluted using a more shallow gradient, 0.048 % (v/v)

Peptide	ε_p	Q value	Q_{rel}
IGF-I	251009	32.82	0.980
long-R^3-IGF-I	305269	33.50	1.000
long-R^3A^{16}-IGF-I	298061	32.99	0.985
long-R^3V^{10}-IGF-I	305269	33.55	1.002
long-R^3L^{25}-IGF-I	298061	32.84	0.980
long-R^3E^{53}-IGF-I	305269	33.45	0.999
long-R^3Q^{59}-IGF-I	308115	33.83	1.010
FVN-R^3-IGF-I	269601	33.54	1.001
FNV-R^3A^{16}-IGF-I	262393	32.96	0.984
FVN-R^3V^{10}-IGF-I	269601	34.45	1.028
FVN-R^3L^{25}-IGF-I	262393	32.73	0.977
FVN-R^3E^{53}-IGF-I	269601	33.49	1.000
FVN-R^3Q^{59}-IGF-I	272447	33.91	1.012

TABLE 3.1
Extinction coefficients and Q values used for estimation of protein.

The extinction coefficients (ε_p : mol^{-1} cm^{-1} at 215 nm) and Q values for the long-R^3-IGF-I and FVN-R^3-IGF-I analogues were calculated using the method described by Buck *et al.* (1989). Q values are the extinction coefficient for the peptide divided by the molecular weight of the protein. Q values relative to long-R^3-IGF-I are shown as Q_{rel}.

acetonitrile/min. Selected fractions were pooled, aliquoted, dried and stored at -20°C. Vials of peptide were quantitated by analytical reverse phase HPLC on a Brownlee microbore C4 column with a linear gradient of 15 to 50 % (v/v) acetonitrile in 0.1 % (v/v) TFA (as described above), using reference recombinant IGF-I and long-R^3-IGF-I as standards. Mass quantitation of peptides was determined by Dr. M. Sheil, Department of Chemistry, University of Wollongong, Wollongong, New South Wales using electrospray mass spectroscopy as described in Section 2.3.2.3.

3.3.6 In vitro characterisation of long-R^3-IGF-I analogues

The biological actions of the long-R^3-IGF-I analogues were determined, on quantitated vials of peptide, using the following *in vitro* biological assays to measure type 1 IGF-receptor binding, protein synthesis stimulation and IGFBP interactions. IGF-I iodinations were performed by Mr S. E. Knowles, CRC for Tissue Growth and Repair, Adelaide, SA, Australia as described in Section 2.3.2.4.

3.3.6.1 Type 1 IGF-receptor binding

The method of Ross *et al.* (1989) was used to measure binding of the analogues to the type 1 IGF-receptor in rat L6 myoblasts, as described in Section 2.3.3.1. This assay was performed three times with triplicate determinations at each peptide concentration.

3.3.6.2 Stimulation of protein synthesis

Stimulation of protein synthesis by the analogues was measured using the method of Francis *et al.* (1986) described in Section 2.3.3.2. This assay was performed four times with triplicate determinations at each peptide concentration.

3.3.6.3 IGFBP competitive binding assay

The relative binding of the peptides to IGFBPs in rat L6 myoblast conditioned medium was measured as described by Francis *et al.* (1992). The IGFBPs were prepared from serum-free DMEM conditioned by confluent monolayers of cells for 24 h. The collected

medium was centrifuged at 10 000 g for 5 min, filtered through a 0.22 μm membrane filter and stored at -20° C prior to usage. Conditioned medium (50 μl) was incubated with ^{125}I-labelled IGF-I and unlabelled peptides in 50 mM phosphate buffer, pH 6.5 + 0.25 % (w/v) BSA, in a total volume of 250 μl, for 18 h at 4° C. Free ^{125}I-labelled IGF-I was separated from IGFBP-bound label by incubation with 1 ml of activated charcoal solution (50 mM phosphate buffer, pH 6.5, 0.25 % (w/v) BSA, 0.5 % (w/v) activated charcoal) at 4° C for 30 min followed by centrifugation at 4 000g for 15 min at 4° C. Radioactivity associated with the supernatant was determined and used to calculate the percentage of radioactivity bound to the IGFBPs. Binding was expressed as the percentage of ^{125}I-labelled IGF-I bound in the absence of unlabelled peptide.

3.3.7 Peptide degradation with pepsin and subsequent HPLC analysis

Intact or modified variants of long-R^3-IGF-I were digested in 10 mM HCl with pepsin and samples were analysed on reverse-phase HPLC as described in Section 2.3.1.

3.4 RESULTS

3.4.1 Expression and purification of the long-R^3-IGF-I analogues.

The recombinant long-R^3-IGF-I analogues, long-R^{3}A^{16}-IGF-I, long-R^{3}L^{25}-IGF-I, long-R^{3}V^{10}-IGF-I, long-R^{3}E^{53}-IGF-I and long-R^{3}Q^{59}-IGF-I were produced intracellularly in *E. coli* JM101 when the cells were transformed with the expression vector and expression was induced with IPTG.

In small scale preparations, total cell protein was analysed by gradient polyacrylamide gels (Figure 3.5). In all induced samples, a predominant band was detected at approximately 9 kDa which was consistent with the expected molecular mass for the long-R^3-IGF-I analogues. Electrophoresis of non-induced samples showed only a minor band at the same molecular weight (Figure 3.5).

On a larger scale using 1 litre fermentations, the production levels of inclusion bodies were from 0.5 to 1.3 g wet weight per litre of growth medium. Downstream processing (as

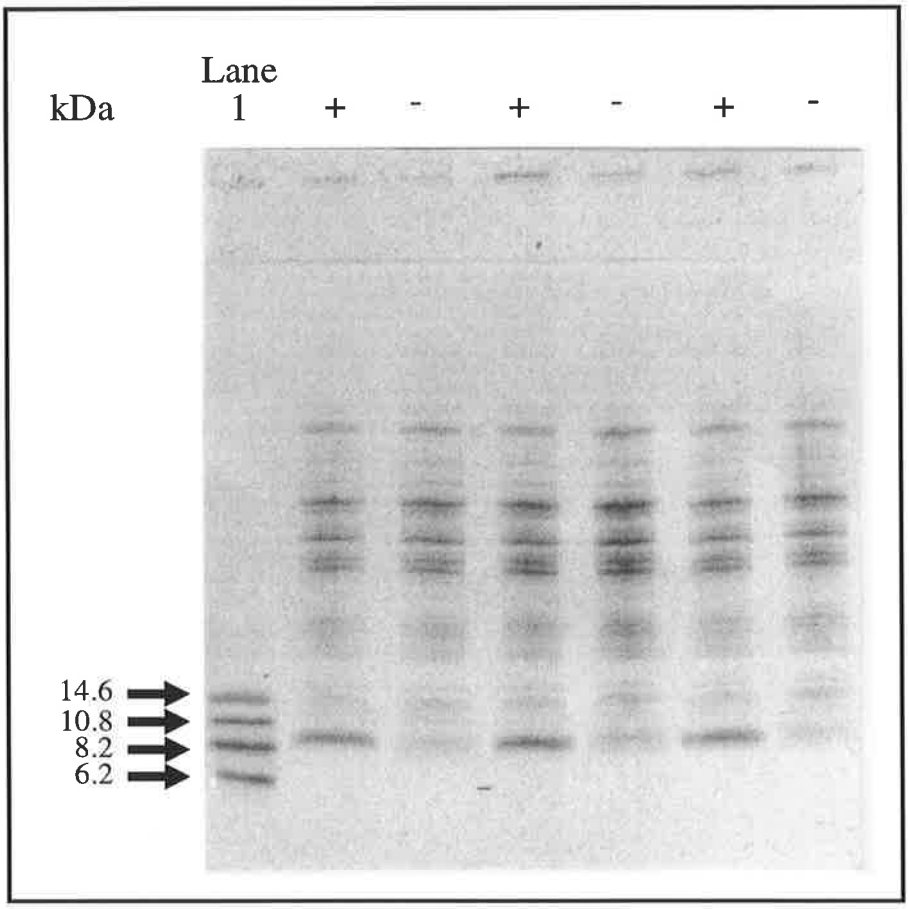


FIGURE 3.5
Polyacrylamide gel analysis of total cell protein from *E. coli* cells induced to express the long- R^3-IGF-I analogues.
Lysed cells from small scale inductions were analysed on gradient 8-12 % polyacrylamide gels under reducing conditions. Molecular weight markers are in lane 1 with their respective molecular masses shown in kDa. (+) indicates cells induced with IPTG and (-) indicates control cells with no induction.

shown in Figure 3.4) for each of the peptides was similar and is described only for long-R^3A^{16}-IGF-I. The inclusion bodies (1.3 g wet weight), dissolved and desalted under reducing conditions in 8 M urea, contained 38.8 mg total protein. The inclusion body proteins were resolved into a major peak (84 % of the total protein) and two minor peaks as shown by analytical reverse-phase HPLC (Figure 3.6a). The subsequent refolding of the reduced long-R^3A^{16}-IGF-I under oxidising conditions resulted in an approximate 100 % yield of oxidised product (Figure 3.6a). Concentration of this major refolded product by FPLC on a C18 silica column followed by ion exchange chromatography produced 29.9 mg of partially pure peptide. Further purification through two reverse-phase HPLC steps yielded 13.0 mg of long-R^3A^{16}-IGF-I peptide which eluted as a single peak on the standard analytical HPLC (Figure 3.6b). This recovery of long-R^3A^{16}-IGF-I peptide corresponded to an overall yield of 33 % relative to the total protein in the inclusion bodies or alternatively, 40 % relative to the putative precursor peak (Figure 3.6a). Electrospray mass spectrometry of the peptide gave the expected mass of 9035 (Figure 3.6c and Table 3.2) confirming that the Phe16Ala mutation had been incorporated into the long-R^3-IGF-I peptide.

Purification of the other long-R^3-IGF-I analogues was similar although the final reverse-phase HPLC gradient was modified depending on the retention time of the mutant. Elution of the analogues, as measured on analytical reverse-phase HPLC, varied from 36.2 % acetonitrile for long-R^3A^{16}-IGF-I to 37.4 % acetonitrile for long-R^3E^{53}-IGF-I (Figures 3.7 and 3.8). The final yields of the analogues ranged from 4.2 mg for long-R^3V^{10}-IGF-I to 14.5 mg for long-R^3E^{53}-IGF-I. Purity was greater than 90 % as assessed by reverse-phase HPLC and mass spectrometry. The chromatograms of both analyses are shown in Figure 3.7 and Figure 3.8 of each analogue, and the calculated and measured molecular masses are indicated in Table 3.2.

Purified long-R^3V^{10}-IGF-I was found to contain additional minor peaks. The fractions containing the long-R^3V^{10}-IGF-I peptide from the final reverse-phase HPLC purification step were freeze-dried prior to aliquotting. When this peptide was reconstituted in 10 mM acetic acid and analysed by standard analytical HPLC before aliquotting and redrying, an additional two minor peptide peaks were present (Figure 3.9a) representing approximately 5 % of the

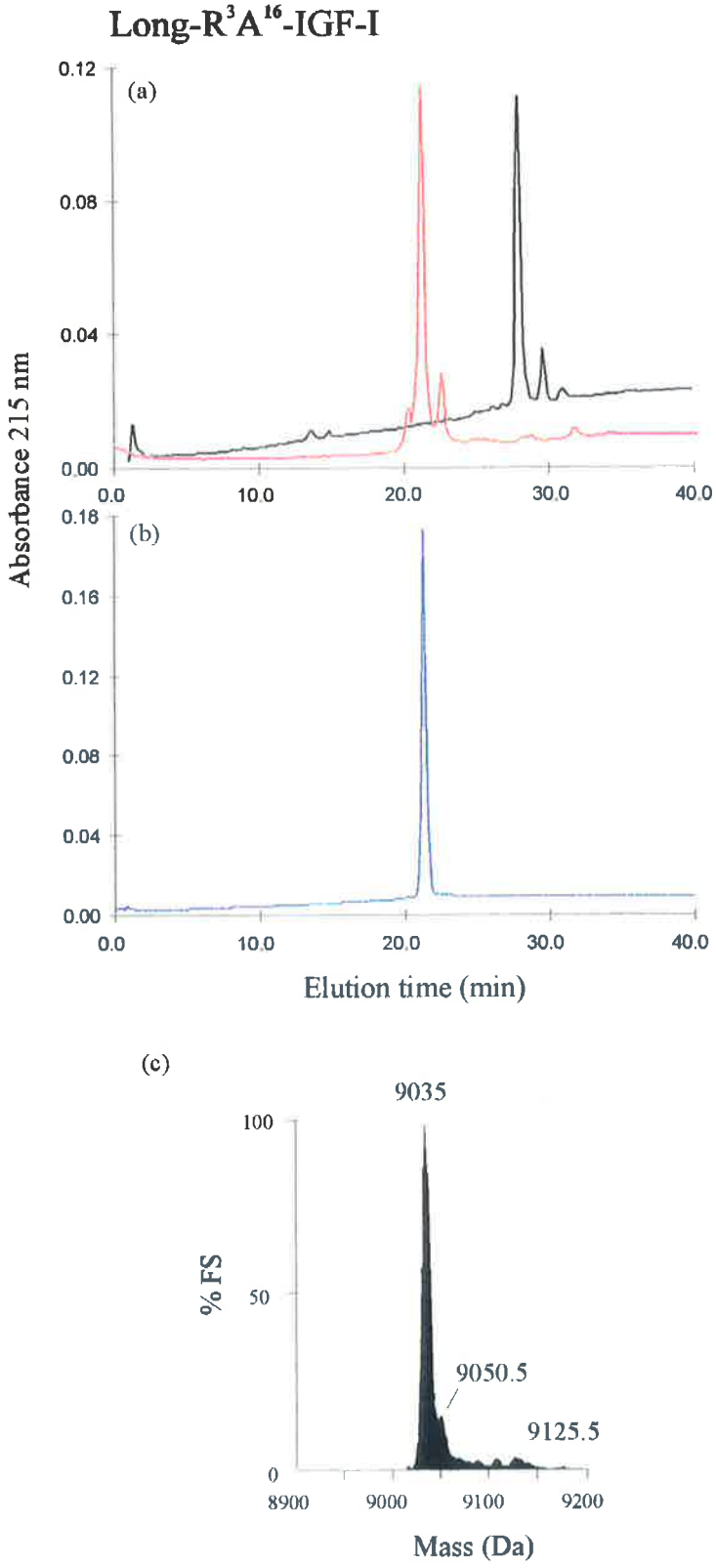


FIGURE 3.6
Production of recombinant long-R^{3}A^{16}-IGF-I
Inclusion bodies from *E. coli* expressing the long-R^{3}A^{16}-IGF-I construct were solubilised in denaturing buffer and desalted by chromatography on a Sephadex G-25M column. (a) Analysis on a microbore C4 reverse-phase HPLC column as described in Section 3.3.2.1 is shown before (red line) and after (black line) the refolding reaction. (b) The long-R^{3}A^{16}-IGF-I peptide after purification to homogeneity. (c) Electrospray mass analysis of long-R^{3}A^{16}-IGF-I. Numbers indicate the masses of the peaks in Daltons.

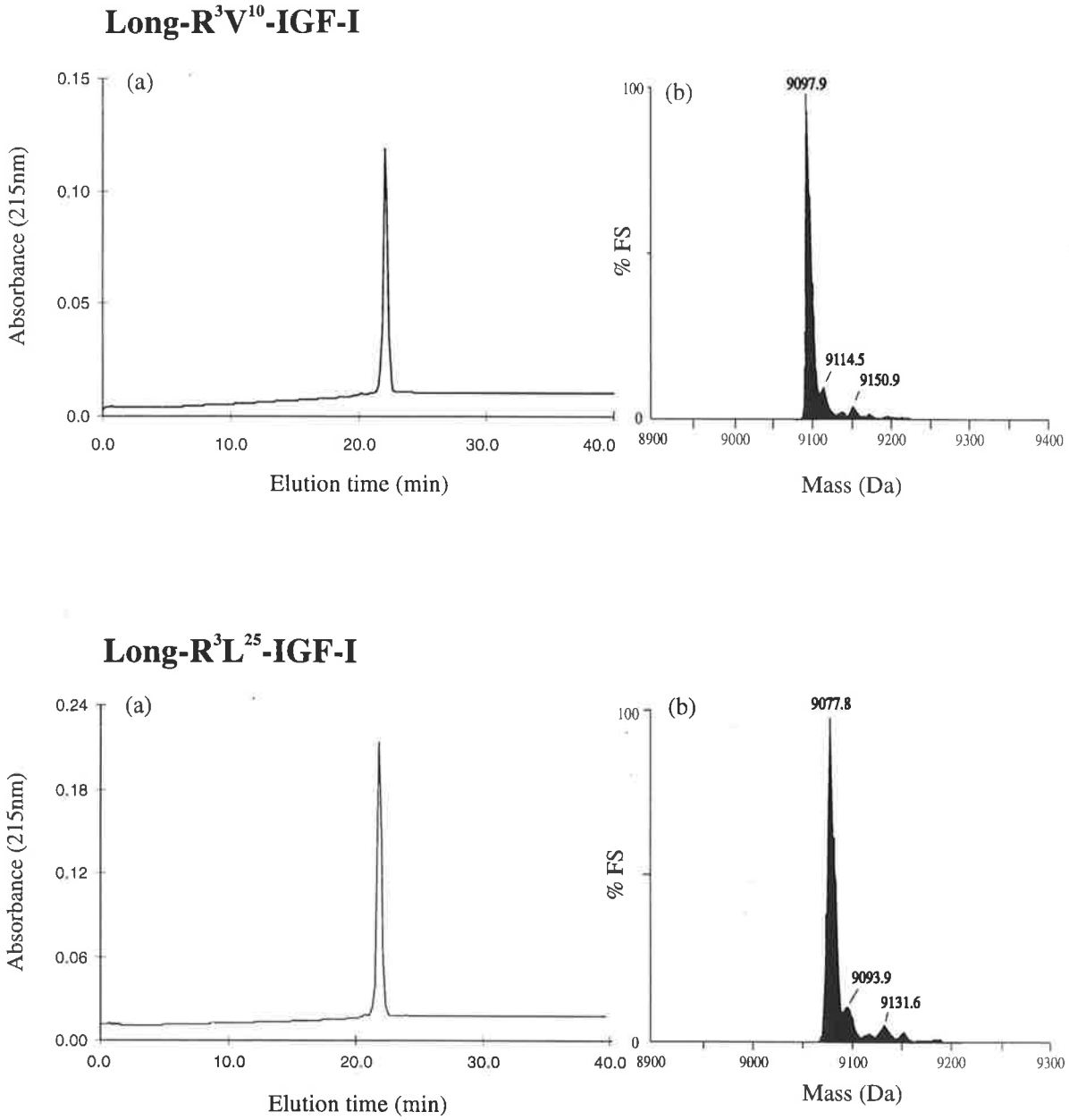


FIGURE 3.7
Purified recombinant long-R^3V^{10}-IGF-I and long-R^3L^{25}-IGF-I.
(a) The long-R^3V^{10}-IGF-I and long-R^3L^{25}-IGF-I peptides after purification to homogeneity as analysed on a microbore C4 reverse-phase HPLC column as described in Section 3.3.2.1. Long- R^3V^{10}-IGF-I eluted at 37.1 % acetonitrile in 0.1 % (v/v) TFA and long-R^3L^{25}-IGF-I eluted at 36.8 % acetonitrile in 0.1 % (v/v) TFA.
(b) Electrospray mass analysis of long-R^3V^{10}-IGF-I and long-R^3L^{25}-IGF-I. Numbers indicate the masses of the peaks in Daltons.

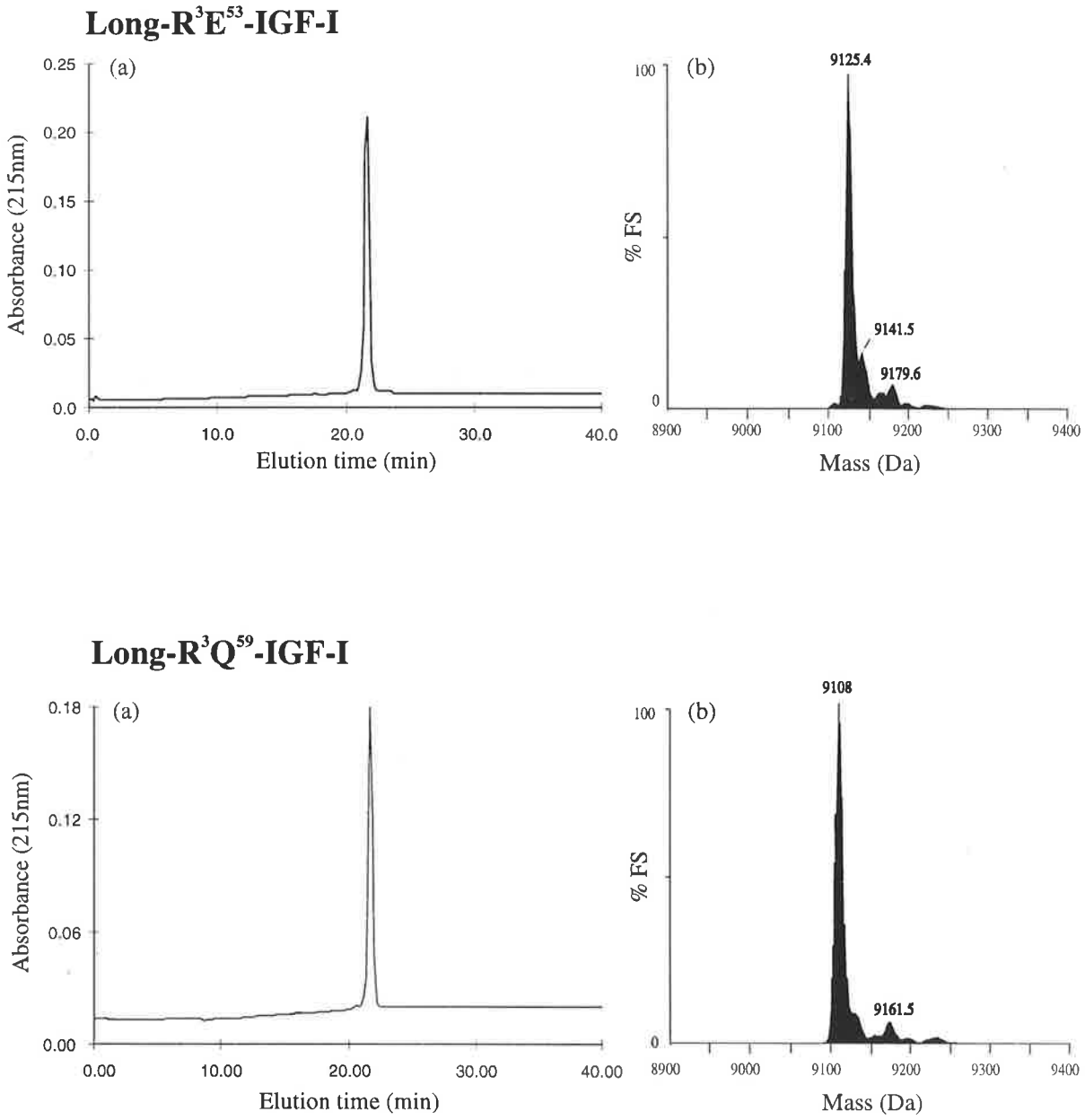


FIGURE 3.8
Purified recombinant long-R^3E^{53}-IGF-I and long-R^3Q^{59}-IGF-I.
(a) The long-R^3E^{53}-IGF-I and long-R^3Q^{59}-IGF-I peptides after purification to homogeneity as analysed on a microbore C4 reverse-phase HPLC column as described in Section 3.3.2.1. Long-R^3E^{53}-IGF-I eluted at 37.4 % acetonitrile in 0.1 % (v/v) TFA and long-R^3Q^{59}-IGF-I eluted at 36.8 % acetonitrile in 0.1 % (v/v) TFA.
(b) Electrospray mass analysis of long-R^3E^{53}-IGF-I and long-R^3Q^{59}-IGF-I. Numbers indicate the masses of the peaks in Daltons.

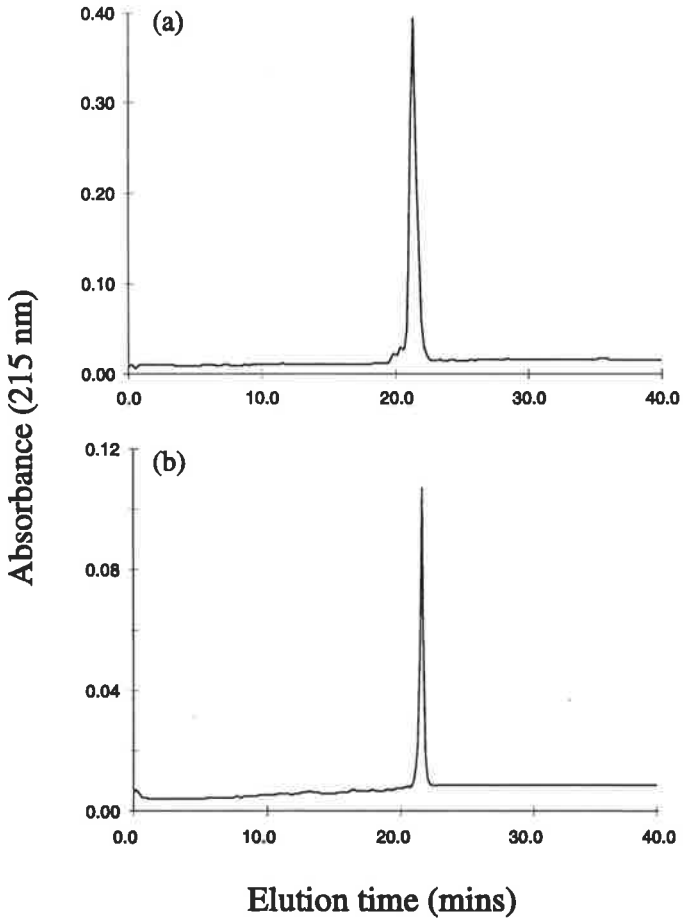


FIGURE 3.9
Possible oxidation of long-R^3V^{10}-IGF-I analogue.
(a) The long-R^3V^{10}-IGF-I peptide analysed by standard analytical HPLC, as described in Section 3.3.2.1, following reconstitution in 10 mM acetic acid after freeze-drying. The presence of two minor peaks are shown which eluted in lower acetonitrile concentrations than the long-R^3V^{10}- IGF-I peptide.
(b) The apparently homogeneous long-R^3V^{10}-IGF-I peptide after an additional reverse-phase HPLC purification step.

Peptide	Molecular mass (Da)	
	Calculated	Actual
long-R^3A^{16}-IGF-I	9035.0	9034.8 ± 0.51
long-R^3V^{10}-IGF-I	9097.0	9097.9 ± 0.55
long-R^3L^{25}-IGF-I	9077.1	9077.5 ± 0.58
long-R^3E^{53}-IGF-I	9125.1	9125.2 ± 0.08
long-R^3Q^{59}-IGF-I	9107.9	9107.9 ± 0.30

TABLE 3.2
Calculated and actual molecular masses of the long-R^3-IGF-I analogues. The calculated molecular mass for the long-R^3-IGF-I analogues were based on a molecular mass of 9111.1 Da for long-R^3-IGF-I. The molecular masses are expressed in daltons (Da).

total protein. Consequently the final reverse-phase HPLC step was repeated in an attempt to remove the contaminating peaks. As peptide fractions pooled at the end of this procedure showed an apparently homogeneous peptide (Figure 3.9b), samples were aliquoted and dried. Subsequent quantitation of the long-R^3V^{10}-IGF-I peptide reconfirmed the presence of these minor peaks at concentrations of less than 2 % of the total protein. Similar minor peaks were also noted during the quantitation of the other long-R^3-IGF-I analogues. These peaks may be associated with oxidation of the peptides which most likely occurred during the drying process. Evidence for oxidation is indicated by the mass spectrometry data for the analogues (Figures 3.6c, 3.7c and 3.8c) where the presence of a peak is shown with a molecular mass of 16 Da higher than that expected for each analogue. Further, Forsberg *et al.* (1990) isolated and characterised modified variants of recombinant IGF-I which included a variant where the methionine residue had been oxidised to methionine sulphoxide. As these peaks in the long-R^3-IGF-I analogues were present at such low concentrations their presence was assumed to have a minimal effect when determining pepsin resistance and biological activity of the analogues.

3.4.2 Biological activities of the long-R^3-IGF-I analogues

In order to characterise the impact the different mutations had on the function of the long-R^3-IGF-I molecule, the biological activities of the different analogues were measured. This was performed in three different assay systems which measure protein synthesis stimulation in L6 myoblasts and affinities for the type 1 IGF-receptor and IGFBPs.

3.4.2.1 Stimulation of protein synthesis by the long-R^3-IGF-I analogues

The ability of the recombinant long-R^3-IGF-I analogues to stimulate protein synthesis in L6 rat myoblasts was compared to that of long-R^3-IGF-I and IGF-I (Figure 3.10). The five long-R^3-IGF-I analogues stimulated protein synthesis giving maximal responses approximately equivalent to those observed with 5 % (v/v) fetal bovine serum (180 % stimulation above the rate of protein synthesis in serum-free medium). The potency of the analogues was defined as the concentration required to give 50 % of the response observed

with 5 % (v/v) fetal bovine serum. The analogues long-R^3Q^{59}-IGF-I and long-R^3E^{53}-IGF-I were slightly more potent than long-R^3-IGF-I, so that half-maximal stimulation of protein synthesis was obtained at approximately 1.6 ng/ml of the analogues compared with 2.0 ng/ml of long-R^3-IGF-I. In comparison, long-R^3A^{16}-IGF-I showed a slight decrease in potency with a half-maximal response at 3.0 ng/ml. Long-R^3V^{10}-IGF-I and long-R^3L^{25}-IGF-I showed significantly decreased levels of protein synthesis stimulation compared to the parent molecule, with half-maximal responses at 6.0 ng/ml and 12 ng/ml respectively. Notwithstanding this, the two analogues retained bio-activity comparative with the native growth factor, IGF-I, which showed a half-maximal stimulation of 11 ng/ml (Figure 3.10).

3.4.2.2 The type 1 IGF-receptor affinity of the long-R^3-IGF-I analogues

Affinity for the type 1 IGF-receptor in L6 myoblasts was assessed by measuring competition for ^{125}I-labelled hIGF-I binding (Figure 3.11). As expected from previous studies (Francis et al., 1992), long-R^3-IGF-I showed a reduced type 1 IGF-receptor affinity compared to the native IGF-I. This was further reduced in some, but not all, of the mutated analogues. Both long-R^3V^{10}-IGF-I and long-R^3L^{25}-IGF-I showed a marked decrease in type 1 IGF-receptor affinity, with half-maximal effects observed at 100 ng/0.5 ml and 20 ng/0.5 ml respectively compared with long-R^3-IGF-I (4.8 ng/0.5 ml). Long-R^3A^{16}-IGF-I, long-R^3Q^{59}-IGF-I and long-R^3E^{53}-IGF-I had receptor affinities similar to long-R^3-IGF-I with half-maximal effects measured at approximately 4.8 ng/0.5 ml.

3.4.2.3 Binding protein interactions of the long-R^3-IGF-I analogues

The affinities of the analogues for the IGFBPs secreted by rat L6 myoblasts were measured by competition for binding of ^{125}I-labelled IGF-I (Figure 3.12). It was observed that long-R^3A^{16}-IGF-I and long-R^3V^{10}-IGF-I had a significantly reduced affinity for these IGFBPs and no binding was noted, even at 10 000 ng/250 µl of added peptide (approximately 4.5 µM). In comparison, the concentration of long-R^3-IGF-I required for a 40 % reduction in tracer binding was 2 700 ng/250 µl (1.19 µM). Long-R^3L^{25}-IGF-I and long-R^3Q^{59}-IGF-I, had a similar affinity for the IGFBPs to that of long-R^3-IGF-I with values

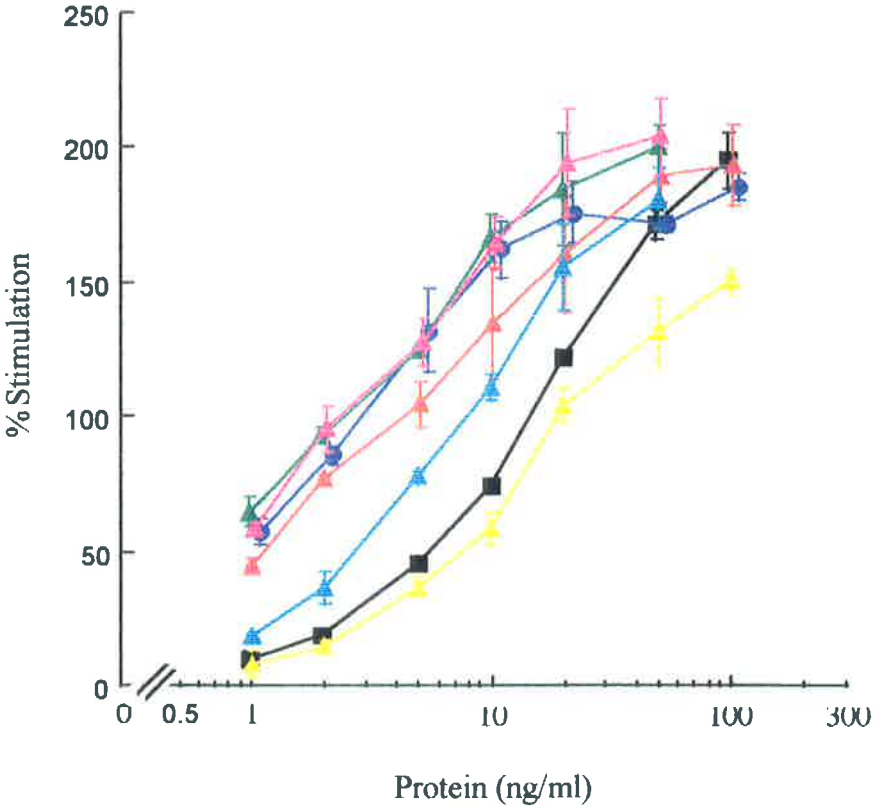


FIGURE 3.10
Biological effects of the long-R^3-IGF-I analogues on protein synthesis in rat L6 myoblasts.
The proteins tested were: human IGF-I (■)
long-R^3-IGF-I (●)
long-R^{3}A^{16}-IGF-I (▲)
long-R^{3}V^{10}-IGF-I (▲)
long-R^{3}L^{25}-IGF-I (▲)
long-R^{3}E^{53}-IGF-I (▲)
long-R^{3}Q^{59}-IGF-I (▲)
Values are the means of triplicate determinations at each peptide concentration. SEM values are indicated by the bars.

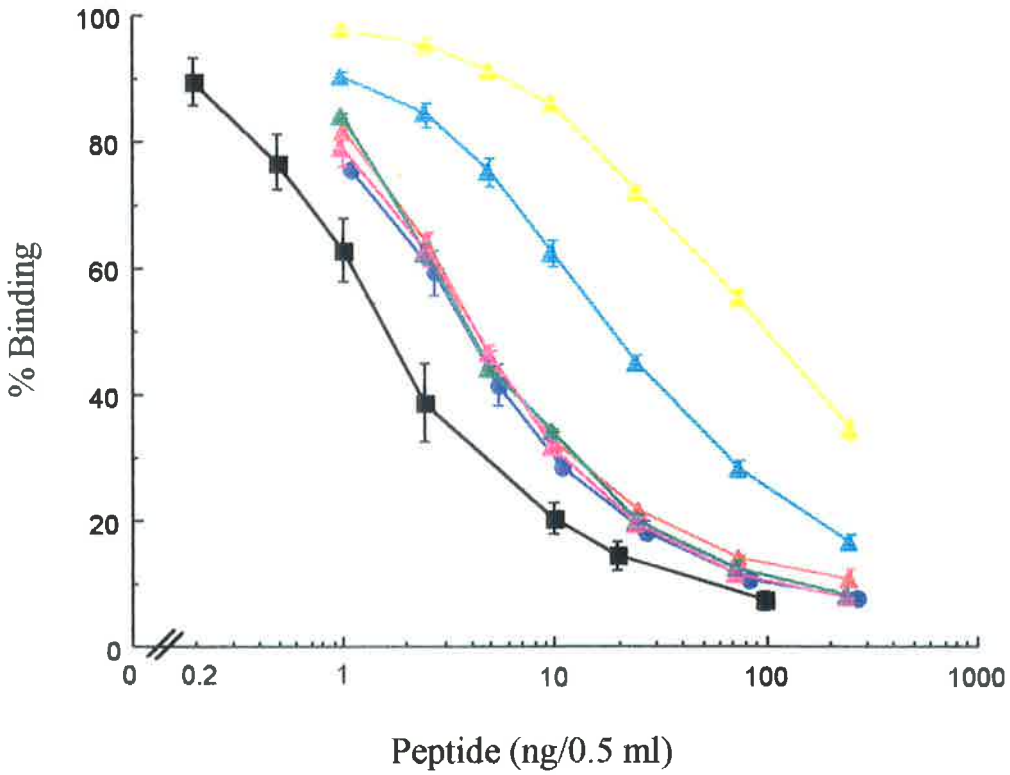


FIGURE 3.11
Binding of the long-R^3-IGF-I analogues to type 1 IGF-receptors in rat L6 myoblasts.
The proteins tested were: human IGF-I (■)
long-R^3-IGF-I (●)
long-R^{3}A^{16}-IGF-I (▲)
long-R^{3}V^{10}-IGF-I (▲)
long-R^{3}L^{25}-IGF-I (▲)
long-R^{3}E^{53}-IGF-I (▲)
long-R^{3}Q^{59}-IGF-I (▲)
Values are the means of triplicate determinations on three separate cultures at each peptide concentration. SEM values are indicated by the bars.

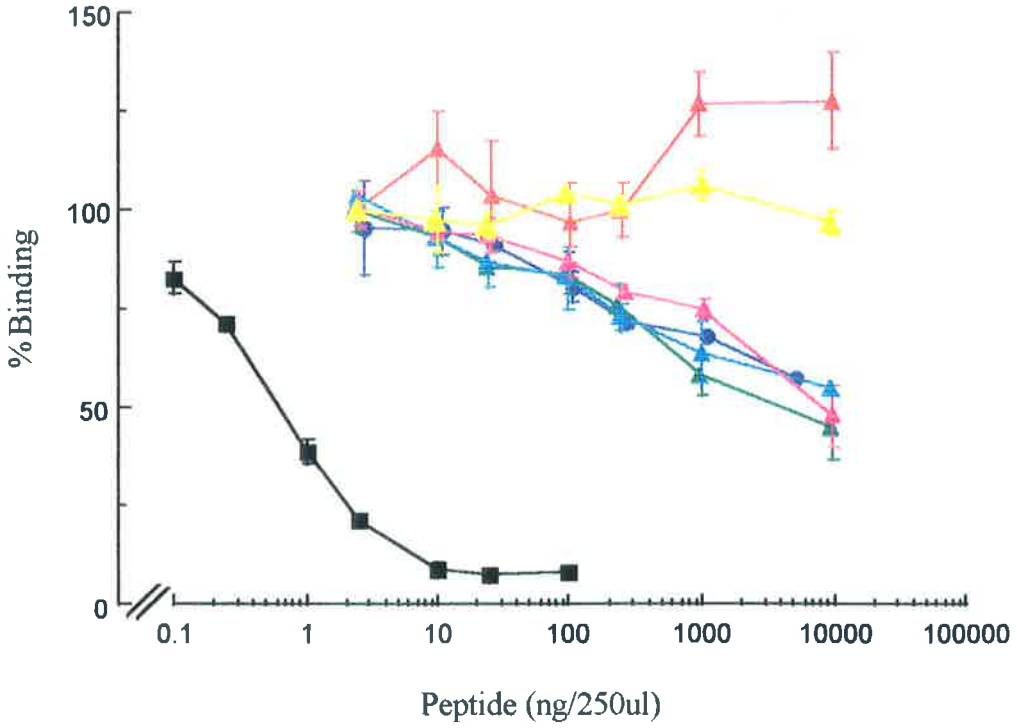


FIGURE 3.12
Binding of the long-R^3-IGF-I analogues to IGF-binding proteins in rat L6 conditioned media.
The proteins tested were: human IGF-I (■)
long-R^3-IGF-I (●)
long-R^{3}A^{16}-IGF-I (▲)
long-R^{3}V^{10}-IGF-I (▲)
long-R^{3}L^{25}-IGF-I (▲)
long-R^{3}E^{53}-IGF-I (▲)
long-R^{3}Q^{59}-IGF-I (▲)
Values are the means of triplicate determinations at each peptide concentration. SEM values are indicated by the bars.

Assay	Mutations					long-R³-IGF-I	IGF-I
	Leu10Val	Phe16Ala	Phe25Leu	Asp53Glu	Met59Gln		
Protein Synthesis	16	64	30	133	114	100	16
Type 1 receptor binding	4	92	20	101	94	100	220
IGFBP binding	<25	<25	93	258	90	100	>1000

TABLE 3.3

Biological properties of the long-R³-IGF-I analogues expressed as a potency relative to long-R³-IGF-I.

The values are shown as the concentration of peptide required to achieve ED_{50} in the various assays, expressed as a percentage of long-R³-IGF-I concentrations. The exception is binding to the IGFBPs which was determined using the concentration of peptide required for a 40 % reduction in tracer binding. Protein synthesis stimulation and receptor binding were determined in rat L6 myoblasts, and IGFBP affinity was measured using rat L6 moblast conditioned media.

of 2 900 ng/250 µl and 3 000 ng/250 µl respectively, while long-R^3E^{53}-IGF-I showed an approximate 2.5-fold greater affinity with 1 050 ng/250 µl of peptide required to give a 40 % reduction in tracer binding. Although the long-R^3E^{53}-IGF-I analogue showed an increased affinity for the IGFBPs, this was still markedly lower than compared that of IGF-I (0.4 ng/250 µl).

A summary of the biological assay data showing the potencies of the long-R^3-IGF-I analogues relative to long-R^3-IGF-I is given in Table 3.3. The Met59Gln mutation had minimal effect on either receptor binding or IGFBP affinity, resulting in little change in bio-activity compared to the parent long-R^3-IGF-I. The only effect of the Asp53Glu mutation was a small increase in IGFBP affinity, with little change in receptor binding and bio-activity. The Phe25Leu mutation reduced receptor binding without modifying IGFBP affinity, causing some reduction in bio-activity. The Phe16Ala mutation resulted in a marked reduction in IGFBP affinity but only slightly compromised receptor binding and bio-activity. The Leu10Val mutation was surprising in that it still retained modest bio-activity despite a substantial loss of receptor binding. This may be related to the marked decrease in IGFBP affinity.

3.4.3 Digestion of long-R^3-IGF-I analogues with pepsin

The purified long-R^3-IGF-I analogues were subjected to pepsin digestion as described in Section 2.3.1. At low enzyme to peptide ratios (w/w) essentially the same peaks were seen in all digests and these eluted at similar acetonitrile concentrations. As observed with long-R^3-IGF-I, the analogues were rapidly degraded at the pepsin-sensitive site in the extension peptide to FVN-R^3-IGF-I analogues, as shown by reverse-phase HPLC (Figure 3.13). The FVN-R^3-IGF-I analogues eluted at times ranging from 20.6 min (27.8 % acetonitrile) for FVN-R^3A^{16}-IGF-I to 24.8 min (29.9 % acetonitrile) for FVN-R^3E^{53}-IGF-I. In all digests, the 10 amino acid peptide which resulted from this first pepsin cleavage eluted at approximately 15 min corresponding to 25 % acetonitrile. The FVN-R^3-IGF-I analogues were then further degraded to smaller peptide fragments, although the degradation rates

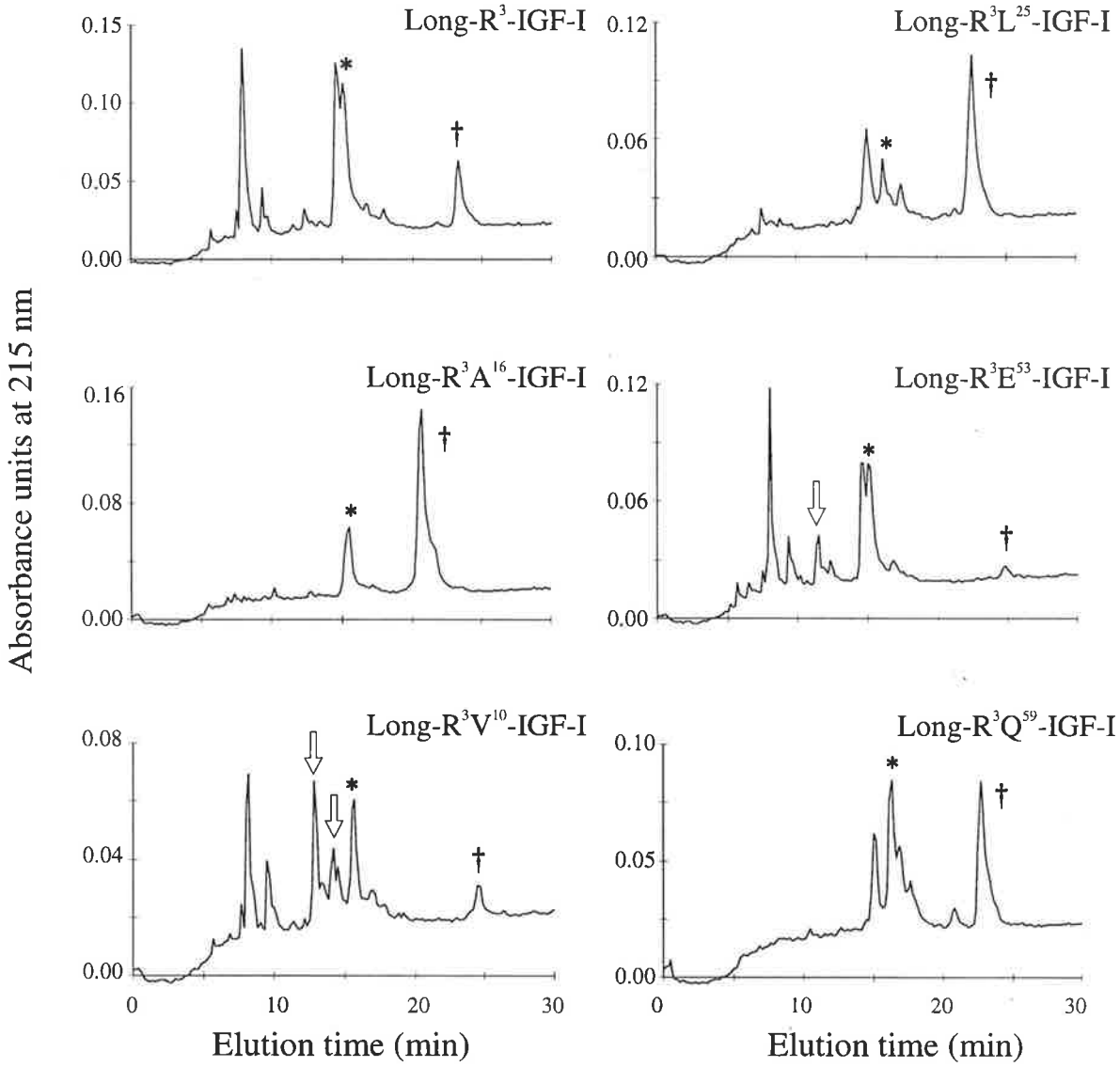


FIGURE 3.13
Comparison of the pepsin digestion of long-R^3-IGF-I and the analogues, analysed by reverse-phase HPLC.
Digestions were performed using a pepsin : substrate ratio of 1 : 10 for 15 min at 37° C. Samples were analysed by HPLC on a Brownlee Aquapore C4 column using a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at A_{215}.† indicates the FVN-R^3-IGF-I analogue, * indicates the ten amino acid *N*-terminal extension fragment, ⇩ indicates peptide fragments not present in the long-R^3-IGF-I degradation. The parent long-R^3-IGF-I molecule and analogues had a retention time of approximately 33 min.

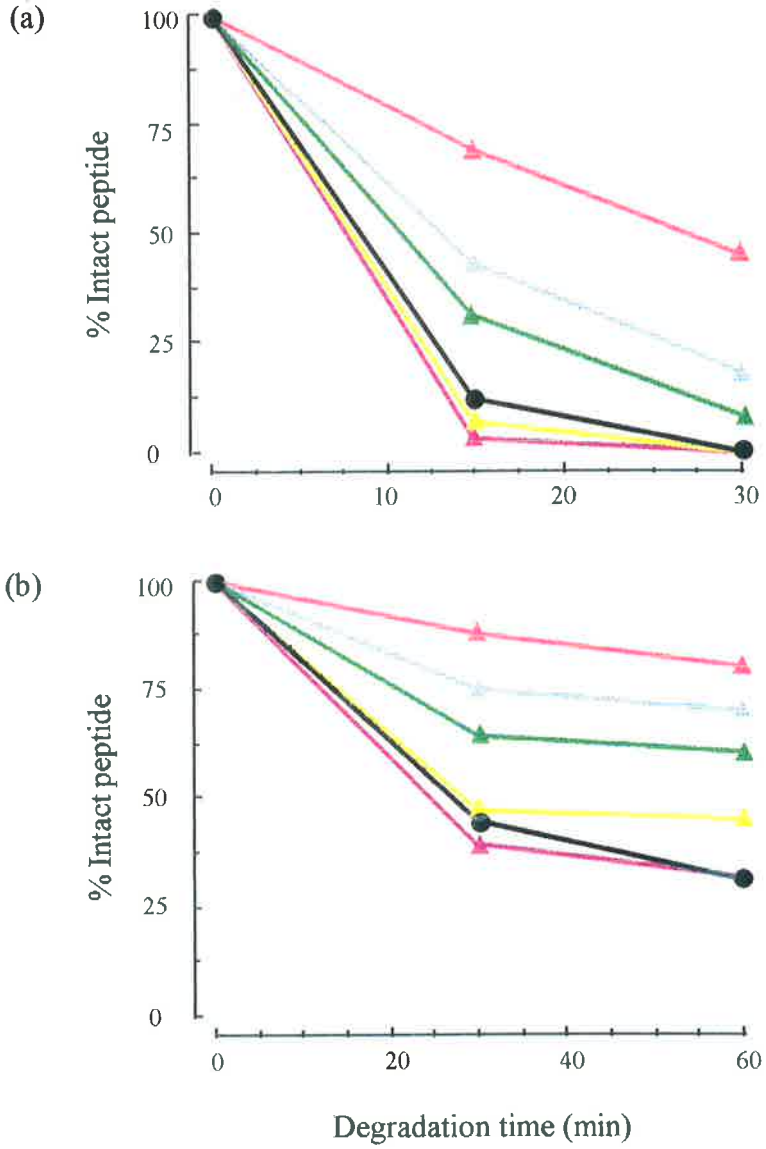


FIGURE 3.14

Comparison of the pepsin degradation rates of long-R³-IGF-I and the analogues, as determined by reverse-phase HPLC.

Digestions were performed using a pepsin : substrate ratio of (a) 1 : 10 and (b) 1 : 30 at 37° C. Values are shown as the cumulative percentage of long-R³-IGF-I analogue and FVN-R³-IGF-I analogue relative to the total protein as measured by area under the peaks on microbore C4 reverse-phase HPLC with a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at A_{215}.

The symbols used are: ● , long-R³-IGF-I;
▲ , long-R³A¹⁶-IGF-I;
, long-R³E⁵³-IGF-I;
, long-R³L²⁵-IGF-I;
▲ , long-R³Q⁵⁹-IGF-I;
▲ , long-R³V¹⁰-IGF-I.

differed markedly between the different analogues. Rates of degradation were deduced by comparing the peptide concentrations determined using the integrated peak area as described in Section 2.3.1 at different time points for the intact analogues relative to the total protein. Intact analogue was assessed as the cumulative percentage of long-R^3-IGF-I and FVN-R^3-IGF-I peptides. As shown in Figure 3.14a, after 15 min degradation at the highest enzyme : substrate ratio (1 : 10), the intact Ala^{16} peptide represented approximately 70 % of the total protein in the degradation compared to 10 % for the parent material, long-R^3-IGF-I, indicating an approximate 7-fold increase in pepsin stability for the long-R^3A^{16}-IGF-I analogue. After 30 min degradation at the same enzyme concentration, the intact Ala^{16} analogue represented approximately 50 % of the total protein whereas no intact parent material remained, suggesting more than 10-fold higher pepsin stability compared with the parent material, long-R^3-IGF-I.

From the digestion experiments, degradation rates of the Ala^{16}, Leu^{25} and Gln^{59} FVN-R^3-IGF-I peptides were markedly reduced compared with long-R^3-IGF-I. An example is shown in Figures 3.13 and 3.14. Long-R^3V^{10}-IGF-I showed a rate of degradation similar to that of long-R^3-IGF-I, whereas long-R^3E^{53}-IGF-I appeared to be degraded at a slightly faster rate (Fig. 3.13 and 3.14). Enzyme : substrate ratios ranging from 1 : 10 to 1 : 100 were investigated with time-points analysed between zero to 60 min. Pepsin degradation data for the long-R^3-IGF-I analogues at a 1 : 30 enzyme to substrate ratio is summarised in Figure 3.14b. For each analogue, a similar fragmentation pattern was obtained between the digestion experiments. The relative degradation rates of the analogues supported the pepsin stability trend noted above (Figures 3.14a and 3.14b).

In summary, long-R^3A^{16}-IGF-I showed the greatest pepsin stability, being stabilised approximately 10-fold compared to long-R^3-IGF-I, as determined by reverse-phase HPLC at different digestion times. Long-R^3L^{25}-IGF-I and long-R^3Q^{59}-IGF-I were stabilised 4- and 3-fold respectively, while long-R^3V^{10}-IGF-I and long-R^3E^{53}-IGF-I were equivalent or slightly less pepsin resistant than long-R^3-IGF-I.

In the more concentrated pepsin digestions, differences were obtained in the fragmentation patterns between the analogues. The patterns of digestion were difficult to

analyse by HPLC alone due to the variation in the eluting acetonitrile concentration of the peptide fragments containing the mutations. However, a further analysis of the reverse phase HPLC chromatograms of the pepsin digestions of long-R^3V^{10}-IGF-I and long-R^3E^{53}-IGF-I suggested the appearance of peptide peaks which were not present in the long-R^3-IGF-I degradation (Figure 3.13). This could indicate that pepsin is cleaving at novel sites in these molecules compared to the parent molecule. These substitutions may have resulted in structural changes to the molecule which expose new sites to the protease or alternatively, pepsin cleavage is inhibited at the modified positions and therefore other bonds or 'minor sites' may have become preferred cleavage sites. To confirm if pepsin cleavage is occurring at 'new' sites, peptide fragments would need to be collected then further analysed by mass spectrometry and/or amino acid sequencing.

3.5 DISCUSSION

In this chapter, single point mutations were constructed in long-R^3-IGF-I and the analogues were characterised with respect to retention of biological activity and resistance to pepsin cleavage. The mutations had been designed as conservative substitutions with amino acids unlikely to disturb protein structure (Bordo & Argos 1991) while also attempting to confer pepsin resistance (Dixon & Webb 1964). Table 3.4 is a summary showing relative pepsin stabilities and biological potencies of the long-R^3-IGF-I analogues compared to the parent material, long-R^3-IGF-I.

3.5.1 Long-R^3V^{10}-IGF-I ; Leu10Val mutation

The Leu10Val mutation showed a marked decrease in binding to the type 1 IGF-receptor and a decrease in protein synthesis stimulation. In addition, affinity for the IGFBPs was markedly reduced. This mutation did not increase the pepsin-stability of long-R^3-IGF-I (Table 3.4).

Substitutions of Leu10 have not been described elsewhere, although the first 16 amino acid residues of the B domain of IGF-I appear to be more critical for binding to all the

Assay	Mutations					long-R³-IGF-I	IGF-I
	Leu10Val	Phe16Ala	Phe25Leu	Asp53Glu	Met59Gln		
Protein Synthesis	16	64	30	133	114	100	16
Type 1 receptor binding	4	92	20	101	94	100	220
IGFBP binding	<25	<25	93	258	90	100	>1000
Pepsin stability	no	10-fold	4-fold	no	3-fold	no	no[a]

TABLE 3.4
Summary of pepsin stability and biological properties of the analogues relative to the parent material, long-R³-IGF-I.
[a] From Xian *et al.* (1995).

IGFBPs than to the receptors (Oh *et al.*, 1993; Bayne *et al.*, 1988). The reduced affinity of this analogue for the IGFBPs was therefore in accordance with previous data. Since there is no evidence for Leu10 being involved directly in receptor binding, it was interesting to see the effect on type 1 IGF-receptor binding. A structural change might therefore account for the significantly decreased receptor affinity reflected in the decreased biological potency. The NMR-structure of IGF-I indicates that Leu10 is in the first helix, an area of defined structure (Cooke *et al.*, 1991; Sato *et al.*, 1993). Further, the side-chain is mostly buried in the hydrophobic core (Cooke *et al.*, 1991) so that substitution at this residue may have altered the structural conformation (both secondary and tertiary) of the peptide. It was surprising that the Val substitution was not as effective as expected, especially as Bordo & Argos (1991) report that a buried Leu residue can be interchangeable with high statistical significance to Val, Ile, Phe or Met. In addition, the side-chain of a Val amino acid covers a similar although slightly smaller spatial area compared to a Leu residue suggesting interference due to steric bulk would be limited. Perhaps the presence of two adjacent Val residues (positions 10 and 11) provided additional steric hindrance and altered the structure of the α-helical region.

The data in this study also showed the Leu10Val mutation did not stabilise the long-R^3-IGF-I molecule. As the Val mutation caused a possible structural change in the peptide, substitution with other bulky amino acids such as Ile, Phe or Met (Bordo & Argos, 1991), some of which are also susceptible to pepsin proteolysis, may cause a similar effect. Consequently an amino acid substitution to Gly or Ala may cause less interference while also conferring pepsin stability. Alternatively, Val11 may be a more appropriate residue for mutation. As the side-chain of this residue is more exposed (Cooke *et al.*, 1991) substitution to other amino acids may be more readily accommodated.

3.5.2 Long-R^{3}A^{16}-IGF-I ; Phe16Ala mutation

Overall, the Phe16Ala substitution was a favourable mutation as it conferred a marked increase in pepsin stability on the parent molecule while not significantly affecting biological activity (Table 3.4).

The mutation Phe16Ala showed a significant decrease in affinity for the IGF binding proteins secreted by rat L6 myoblasts (Table 3.4). In addition type 1 receptor binding remained unaffected although protein synthesis stimulation was slightly decreased. This was a surprising result, as the markedly reduced IGFBP affinity of this analogue might have been expected to increase the bio-activity of the peptide compared to long-R^3-IGF-I. This is shown, for example, by the IGF-I analogue, des(1-3)IGF-I (Bagley *et al.*, 1989; Francis *et al.*, 1993). However, the results from the Phe16Ala mutation may reflect the fact that the IGFBP affinity of long-R^3-IGF-I is so low that a further reduction in affinity had little effect on levels of free peptide during incubation in the rat L6 myoblast bio-assay.

Single site mutations at this Phe16 residue have not been published previously, although a Tyr15-Leu16 IGF-I (based on the analogous insulin residues) has reduced IGF binding protein affinity and increased insulin and type 2 IGF-receptor binding with no alteration in type 1 IGF-receptor binding (Bayne *et al.*, 1988). As the first 16 amino acids of IGF-I have been implicated as being important in the binding of IGFBPs (Bayne *et al.*, 1988; Oh *et al.*, 1993) the reduced IGFBP affinity obtained with the Phe16Ala mutation was not unexpected.

The studies by Oh (Oh *et al.*, 1993) and Bayne (Bayne *et al.*, 1988) have also shown that the first 16 amino acids of the *B* domain of IGF-I appear to be less critical for receptor binding. This is in agreement with the long-R^{3}A^{16}-IGF-I mutant having no significant effect on receptor affinity compared to the parent material (Table 3.4).

3.5.3 Long-R^{3}L^{25}-IGF-I ; Phe25Leu mutation

As with the Leu10Val mutation, the Phe25Leu variant showed a significant impact on affinity for the type 1 receptor and decreased protein synthesis stimulation (Table 3.4). Although point mutations have not been described for Phe25, other data strongly suggests

that the important type 1 receptor binding domain is within residues 24-37 of IGF-I (Cascieri *et al.*, 1991), with the Phe-Tyr-Phe sequence at residues 23-24-25 recognised as playing an important role (Cascieri *et al.*, 1988). These three residues are part of an extended beta turn conformation (Sato *et al.*, 1993; Cooke *et al.*, 1991) and are exposed, lying along a cleft on one face of the IGF-I molecule with their side chains flat across the surface (Cooke *et al.*, 1991). Therefore, a modified receptor binding for the long-R^3L^{25}-IGF-I analogue was in accordance with this data.

Although the Phe25Leu mutation decreased receptor binding, the effect on bio-activity was modest, so that the mutant remained more active then native IGF-I. Moreover, its effective bio-activity in the stomach is likely to be increased as a result of its 4-fold enhanced stability against pepsin digestion. If the biological activity could be further improved by a different mutation then this analogue may have an even greater potential in its usage in the gastrointestinal tract. Based on the matrices proposed by Bordo and Argos (1991), the Phe25Leu mutation is a favourable substitution, regardless of the exposed or buried status of the Phe residue. Recognising, however, that Phe25 is in an exposed position from NMR studies, substitution for Tyr, Thr, Ile, Asn or Lys could be favourable (Bordo & Argos, 1991). As pepsin proteolysis is still likely to occur with either Tyr or Ile at P_1' (Powers *et al.*, 1977), substitution of Phe25 to Thr, Asn or Lys may result in the required pepsin stability as well as retention of biological activity. However, the impact of such a hydrophilic substitution in this hydrophobic surface region of the peptide, would have to be investigated with respect to both structure and function of the IGF molecule. Perhaps a more hydrophobic residue, such as Ala, would be more suitable.

3.5.4 Long-R^3Q^{59}-IGF-I ; Met59Gln mutation

The Met59Gln substitution was also a successful mutation as pepsin stability was increased in the long-R^3Q^{59}-IGF-I analogue while biological activity was effectively retained (Table 3.4).

There is published data of other single site mutations at the Met59 position, specifically, Met59Thr (the analogous residue in IGF-II) and Met59Norleu (Peters *et al.*,

1985; Forsberg *et al.*, 1990). Both these analogues have also been shown to have a minor effect on receptor binding, showing a 20 % and 30 % decreased affinity respectively (Peters *et al.*, 1985; Forsberg *et al.*, 1990). These results combined with the data from long-R^3Q^{59}-IGF-I, suggests that Met59 is not directly involved in type 1 IGF-receptor binding.

As Met59 is very sensitive to oxidation (Forsberg *et al.*, 1990) it is consequently an exposed residue, explaining its accessibility for pepsin cleavage in the native conformation. The mutation Met59Gln did not drastically affect biological activity but substituting the methionine did improve the stability towards pepsin. This is in agreement with Dixon and Webb (1964) who state that a sulphur-containing residue, such as Met, has been found to facilitate the action of pepsin on tyrosine-containing peptides. Consequently, the mutation Met59Gln, removing the methionine residue, would seem to be useful in a putative pepsin-resistant IGF-I molecule.

3.5.5 Long-R^3E^{53}-IGF-I ; Asp53Glu mutation

Although the Asp53Gln mutation was the least effective analogue with respect to pepsin stability, the choice of Glu was a successful residue with regards to effects on biological activity (Table 3.4). The Asp53Glu mutation showed a slight increase in biological activity compared with the parent long-R^3-IGF-I. This increase in bio-activity was unexpected, given the increase in IGFBP affinity with no change in receptor binding. In this assay system, the increased IGFBP affinity would be more likely to decrease, not increase, bio-activity. However, the affinity of long-R^3E^{53}-IGF-I for IGFBPs, as secreted from L6 myoblasts, was still markedly reduced compared to the native IGF-I molecule.

As there are no previously reported studies of mutations at this Asp53, this residue has not been shown to be directly involved in type 1 or type 2 receptor binding. My study suggests that Asp53 is not directly implicated in either type 1 receptor or IGFBP binding. Cooke *et al.* (1991) speculate from their model that Asp53 may be involved in binding to the type 2 IGF-receptor.

Although the Asp53Glu mutation did not significantly affect the biological activities measured, the stability against pepsin degradation was unexpectedly decreased when compared to the parent molecule. The peptide was rapidly cleaved to smaller fragments, some of which appeared to be novel compared with long-R^3-IGF-I digestion (Figure 3.13). These could possibly arise from secondary sites being preferred if cleavage is indeed blocked at the Glu residue. Sequencing of the digestion fragments would confirm where pepsin is cleaving in the long-R^3E^{53}-IGF-I molecule and if cleavage has been prevented at the Asp53-Leu54 bond.

3.5.6 Speculation on a further enhanced pepsin-resistant analogue

The half-life of a protein in the gastrointestinal tract is affected by inactivation or degradation by proteases. For therapeutic use, it can therefore be advantageous to prolong the *in vivo* half-life of peptides by removal of protease sensitive sites. Brinkman *et al.* (1992) have effectively used site-directed mutagenesis in this manner, to improve the stability of exotoxin in the circulation of mice. This stabilised exotoxin is being used to develop therapies to treat cancer and other diseases. I have used a similar approach here to remove protease-sensitive sites in IGF-I. The gastrointestinal tract is one of the most responsive target tissues for IGF-I and potent IGF-I analogues as shown by the presence of IGF-receptors throughout the gut of several species, the expression of IGF peptides particularly in the fetal period, as well as experiments showing selective action *in vivo* on the gut (reviewed Read *et al.*, 1991; Lund, 1994). Therefore an IGF-I molecule or analogue that has an increased half-life resulting from decreased proteolysis could have significance in the treatment of a number of gastrointestinal disorders (Table 1.3).

Encouragingly, three of the mutations, Phe16Ala, Asp53Glu, Met59Gln, did not significantly reduce biological activity and of these, Phe16Ala and Met59Gln, increased the half-life of long-R^3-IGF-I against pepsin proteolysis. The other two mutations, Leu10Val and Phe25Leu, lowered biological activity but Phe25Leu also stabilised the long-R^3-IGF-I molecule. Combining these data, an analogue with several mutations could be designed. A double mutant containing the mutations, Phe16Ala and Met59Gln, should most likely further

increase the stability of long-R^3-IGF-I without significant loss of biological activity. The addition of a third mutation at Phe25 could further enhance stability. Although the Phe25 residue seems to be required for bio-activity, substitution with a residue other than Leu may be more preferable.

3.5.7 Summary

In this chapter site-directed mutagenesis has been used to introduce single point mutations at protease-sensitive sites in the long-R^3-IGF-I peptide, which resulted in increased stability against pepsin digestion. Of the mutants, long-R^3A^{16}-IGF-I was the most effective showing an approximate ten-fold increase in stability compared with the parent material, long-R^3-IGF-I. The increased pepsin stability was also achieved without a significant loss in biological activity, as measured by receptor binding and protein synthesis stimulation, suggesting potential for therapeutic use, allowing applications in the stomach as well as intact passage to the duodenum. However, *in vivo* the stomach is a variable environment due to the presence of salt, other proteins, changes in pH and different isozymes of pepsin in the gastric secretions. Consequently the stability of the long-R^3-IGF-I analogues may differ *in vivo* from that shown with purified pepsin, and this question is addressed in the next chapter.

CHAPTER FOUR
DEGRADATION OF LONG-R^3-IGF-I ANALOGUES BY RAT, HUMAN AND PIG STOMACH FLUSHINGS AND PURIFIED PORCINE PEPSIN

4.1 INTRODUCTION

In the previous chapter I produced variants of long-R^3-IGF-I which had increased stability against pepsin digestion *in vitro* and retained biological activity. Both long-R^{3}V^{10}-IGF-I and long-R^{3}L^{25}-IGF-I had reduced affinity for the type 1 IGF-receptor while long-R^{3}A^{16}-IGF-I, long-R^{3}E^{53}-IGF-I and long-R^{3}Q^{59}-IGF-I had binding similar to that of the parent molecule. Although the long-R^3-IGF-I variants exhibited differences in affinities for the type 1 IGF-receptor, all the variants stimulated protein synthesis, with potency between that obtained with IGF-I and long-R^3-IGF-I, as measured in rat L6 myoblasts.

Digestion of the mutant forms by purified porcine pepsin showed long-R^{3}A^{16}-IGF-I had the greatest pepsin stability, being stabilised approximately ten-fold compared to long-R^3-IGF-I. Long-R^{3}L^{25}-IGF-I and long-R^{3}Q^{59}-IGF-I had a four and three-fold higher stability respectively, while long-R^{3}V^{10}-IGF-I and long-R^{3}E^{53}-IGF-I were equivalent or slightly less pepsin resistant than long-R^3-IGF-I. The increased pepsin stability with retained biological activity observed for several of the variants, particularly long-R^{3}A^{16}-IGF-I, suggests potential for therapeutic use, allowing applications in the stomach as well as intact passage to the duodenum.

In this chapter, I have extended the initial investigation to determine whether the variants could exhibit greater stability, compared with long-R^3-IGF-I, under conditions that more closely reflect the stomach environment *in vivo*. The stability of the peptides may differ *in vivo* from that with purified pepsin, due to the presence of salt, other proteins, changes in pH and different isoenzymes of pepsin in the stomach secretions. Therefore I compared the rates of degradation of long-R^3-IGF-I with that of the range of analogues in rat stomach and duodenal flushings. I further investigated the stability of the most pepsin-resistant variant, long-R^{3}A^{16}-IGF-I, in stomach flushings from human and pig. Finally I compared the stability

of long-R^3-IGF-I and long-R^3A^{16}-IGF-I under a range of conditions including assessing stability in two different pH solutions, 10 mM HCl (pH 2.0) and 50 mM glycine (pH 3.2), determining the effect of salt in these buffers and also the effect of pepsin : peptide ratios on pepsin digestion.

4.2 MATERIALS

Long-R^3-IGF-I (receptor grade) peptide was provided by GroPep Pty. Ltd., Adelaide, Australia. ^{14}C-labelled rainbow markers was obtained from Amersham Australia Pty Ltd., Sydney, Australia. High molecular weight markers were from Pharmacia Biotech Sydney, NSW, Australia. Disuccinimidyl suberate was obtained from Pierce Pty Ltd., Rockford, IL, U.S.A and dimethyl sulphoxide from BDH Chemicals, Merck Pty. Ltd., Kilsyth, VIC, Australia. Porcine pepsin (EC 3.4.23.1) and the peptide inhibitors, aprotinin, pepstatin and phenylmethylsulfonyl fluoride (PMSF), were purchased from Boehringer Mannheim Australia (Sydney). Bovine serum albumin (BSA: radioimmunoassay grade), bovine haemoglobin, Coomassie Brilliant Blue-R250, Folin Ciocalteau reagent and β-mercaptoethanol were obtained from Sigma (St Louis, MO, U.S.A.). All other reagents were of analytical grade.

4.3 METHODS

All techniques were performed by myself unless otherwise stated.

4.3.1 Degradation of long-R^3-IGF-I and analogues in gut flushings

4.3.1.1 Collection of gut flushings.

Rat gut flushings were collected by a modified method of Xian *et al.* (1995). Male Sprague-Dawley rats (180-210 g) were fasted 24 h, anaesthetised with brietal/nembutal (9 : 1) and the stomach exposed with a mid-line incision. With minimal handling, the stomach was ligated first at the duodenal end to prevent reflux of the intestinal gastric secretions, then ligated at the oesophageal end. The stomach was excised then flushed with 5 ml of a glycine buffer containing 50 mM glycine, pH 3.2, with 154 mM NaCl and 0.1% (w/v) BSA. The duodenal segment (5 cm distal duodenum plus 1 cm jejunum) was flushed with 5 ml of

154 mM NaCl. Flushings from 3 rats were pooled, centrifuged for 30 min at 4 000 g at 4° C and then stored at -80° C, prior to usage.

Porcine stomach flushings were collected from a male Large White pig (30 kg) after 24 h fasting. The pig was anaesthetised via intra-muscular injection (ketamine hydrochloride) and anaesthesia was maintained using halothane. The stomach was ligated and excised as described above. Gastric secretion present in the stomach was collected, then the stomach was flushed with two 45 ml batches of glycine buffer. The gut flushings were pooled, centrifuged for 20 min at 4 000 g at 4° C, then the supernatant was aliquoted and stored at -80°C. Pig flushings were collected by Dr. J. C. Wallace (CRC for Tissue Growth and Repair, Adelaide, South Australia) and Dr. R. Van Barneveld (Northfield Pig Research Unit, Adelaide, South Australia)

Human stomach flushings were collected endoscopically from an adult female. The flushings were centrifuged, diluted four-fold with glycine buffer, aliquoted and stored as described for the porcine flushings. The human stomach flushings were collected from myself by Dr Richard Holloway, Royal Adelaide Hospital, Adelaide, South Australia.

4.3.1.2 Determination of pepsin activity.

Peptic activity against denatured haemoglobin was measured in the human, rat and pig stomach flushings to standardise them for proteolytic activity against a substrate unrelated to IGF-I. Peptic activity was estimated using a modification of the method of Anson (1938). Bovine haemoglobin (0.4 g) was dissolved in 20 ml of water then filtered through pyrex glass wool. The filtrate was adjusted to pH 1.8 by the addition of 0.3 M HCl. A 250 µl aliquot of the denatured haemoglobin solution was warmed to 37° C before a 50 µl sample containing stomach flushings or purified porcine pepsin (10 to 100 µg/ml) was added. The reaction was terminated at different time points by the addition of 500 µl of 5 % (w/v) TCA. Samples were then centrifuged for 10 min at 14 000 g and the absorbance of the resulting supernatant was measured at 280 nm. Blank determinations were carried out as above except TCA was added to the haemoglobin solution prior to the addition of the enzyme samples.

4.3.1.3 Degradation experiments

The stability of long-R^3-IGF-I and the variants was examined in gut flushings, using either ^{125}I-labelled or unlabelled peptides.

The ^{125}I-labelled long-R^3-IGF-I analogues were prepared as described previously (Section 2.3.2.4) although the iodination reactions were shortened from one minute to 40 seconds. Iodinations were performed by Dr. C. J. Xian, CRC for Tissue Growth and Repair, Adelaide, SA, Australia.

The stability of the full range of long-R^3-IGF-I analogues in gut flushings was assessed using ^{125}I-labelled peptides. In these experiments, 800 µl of stomach flushings were incubated at 37° C with 320 µl of ^{125}I-labelled long-R^3-IGF-I or analogue (5 ng, 560 000 c.p.m.), 0.05 % (w/v) aprotinin and 1 mM PMSF. At different time points, degradation was stopped by taking 200 µl samples into 300 µl of stopping buffer (0.1 M Tris, pH 8.0, containing 154 mM NaCl). Degradation in duodenal flushings was as described above except the protease inhibitors were present in the stopping buffer (0.1 M sodium acetate, pH 4.0, with 0.05 % (w/v) aprotinin and 1 mM PMSF). Control incubations containing no stomach or duodenal flushings were included. These were identical to the test incubations except that the flushings were replaced with 800 µl glycine buffer, and the stopping buffer was added immediately. A time zero control was also included where the stopping buffer was added prior to the addition of the luminal flushings. Samples were assayed using IGF-receptor precipitation and/or TCA assays.

For measurements of the degradation rates of unlabelled long-R^3-IGF-I and long-R^3A^{16}-IGF-I, stomach flushings (650 µl) containing 0.05 % (w/v) aprotinin and 1 mM PMSF were added to 260 ng peptide (475 µl) and incubated at 37° C. The digestion was stopped at different time points by adding 150 µl aliquots to 100 µl of 0.1 M Tris, pH 7.4, containing 0.5 % (w/v) BSA. Samples were then assayed in triplicate using a competitive IGF-receptor binding assay. Pepstatin (1 µM) was included in the incubation mixture in some experiments to determine the proportion of proteolytic activity attributable to pepsin.

4.3.2 Degradation of long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I with purified porcine pepsin

Stabilities of iodinated peptides were measured by determining the half-life in the presence of purified porcine pepsin. These experiments followed an identical protocol to that described for stomach flushings, but porcine pepsin in HCl or glycine buffer was substituted for the flushings. The protease inhibitors, aprotinin and PMSF, were not included in the incubation mixture.

The stabilities of unlabelled long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I to purified porcine pepsin were initially determined using the following method. Pepsin digests were set up in siliconised tubes, with 1273 µl of 10 mM HCl containing 380 ng of peptide. Two aliquots containing 40 ng of peptide were removed and added to 100 µl of 0.1 M Tris (pH 7.4) containing 0.5 % (w/v) BSA. These samples were used for the no pepsin control and the zero time point. A 16 µl aliquot of 10 mM HCl and pepsin (800 ng) were added respectively to each tube which was then stored on ice before assaying for peptide concentration. The remaining aliquot was then temperature equilibrated at 37° C prior to the addition of 104 µl of pepsin (5 200 ng), giving an enzyme : substrate ratio of 20 : 1 (w/w). As the analogues were at a lower concentration in these pepsin digestions, compared to the digests analysed by HPLC (Section 4.3.5 and Chapters 2 & 3), it was necessary to use a higher enzyme : substrate ratio for these experiments. At time points ranging between 5 and 90 minutes, samples of 150 µl (40 ng peptide) were added to 100 µl of ice-cold 0.1 M Tris, pH 7.4, containing 0.5 % (w/v) BSA on ice to stop the reaction. These samples were then assayed in triplicate in a competitive IGF-receptor binding assay (Section 4.3.4)

The stabilities of long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I to porcine pepsin degradation were compared in two different buffers, in the presence and absence of salt. The effect of a range of pepsin : peptide ratios on peptide degradation was also examined. Pepsin digestions of long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I were performed as described above with the following modifications which were used to limit peptide adherence to surfaces.

To investigate the importance of pH buffering on the relative stability of the peptides, pepsin degradation rates of long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I were compared in 10 mM

HCl, pH 2.0, and 50 mM glycine, pH 3.2. The peptides (2.2 µg) were incubated with pepsin, using an enzyme : substrate ratio of 1 : 2.5 (w/w), in a total volume of 825 µl in HCl or glycine buffer. After incubation at 37° C for 5, 15, 30, 60 and 120 min, 150 µl samples of the reaction mix were added to 2.35 ml of ice cold 0.1 M Tris, pH 7.4, containing 0.5 % (w/v) BSA.

The influence of salt concentrations on peptide stability was investigated by comparing the stability of long-R^3-IGF-I and long-R^3A^{16}-IGF-I in the presence and absence of saline, 154 mM NaCl. Incubations were performed in either 10 mM HCl, pH 2.0, or 50 mM glycine buffer, pH 3.2, as described above.

To determine the effect of pepsin : peptide ratios on stability of the variants, enzyme to peptide weight ratios of 20 : 1, 4 : 1 and 1 : 2.5, corresponding to molar ratios of approximately 5 : 1, 1 : 1 and 1 : 10, respectively, were used. Incubations were performed in 10 mM HCl, pH 2.0, and other methodological details were as described above.

4.3.3 Preparation of human placental membrane receptors

4.3.3.1 Isolation of membrane receptors

Human placental membrane receptors were used as a source of type 1 IGF-receptors. Placental receptors were isolated by differential centrifugation in 0.25 M sucrose using the method of Cuatrecasas, (1972). The placenta was dissected free of chorion and blood vessels then homogenised on ice in 0.25 M sucrose with 25 mM Tris, pH 7.4. Homogenised material was filtered through cheesecloth followed by centrifugation at 600 g for 10 min then 10 000 g for 30 min at 4° C. The supernatant was adjusted to 0.1 M NaCl and 0.2 mM $MgSO_4$ and centrifuged at 49 000 g for 40 min at 4° C. The pellet was resuspended in approximately 150 ml of 50 mM Tris, pH 7.4, homogenised, recentrifuged and resuspended in 50 ml of Tris buffer. Membranes were aliquoted and stored at -80° C prior to usage.

4.3.3.2 Protein content determination of membrane receptors

The protein content of the placental membrane receptors was estimated using a modified method of Lowry *et al.* (1951). A 200 µl aliquot of membranes was digested with

an equivalent volume of 1 M NaOH for 2.5 h at 4° C followed by dilution in 1 M NaOH, over a range of 2 to 20-fold. BSA standards (0 to 200 µg/ml) were prepared in 0.5 mM NaOH. Samples of 50 µl (n=8 for standards, n=4 for membranes) were added to wells in a Falcon Microtect III tissue culture plate. A 150 µl aliquot of 2.56 % (w/v) Na_2CO_3, 0.256 % (w/v) sodium tartrate, 0.128 % (w/v) $CuSO_4$ was added to the samples followed by 50 µl of a 25 % (v/v) Folin Ciocalteau solution exactly 10 min later. The reaction was incubated at room temperature for 60 min and the absorbance was read at 690 nm.

4.3.3.3 Cross-linking of IGF analogues to membrane proteins

Cross-linking of human placental membranes to ^{125}I-labelled long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I was based on the method of Kasuga *et al.* (1981) and Drakenberg *et al.* (1993). Membranes (250 µg) were added to 50 mM Tris, pH 7.4, containing 0.5 % (w/v) BSA and 2 mM PMSF giving a total volume of 250 µl. Membrane preparations were incubated in the presence and absence of 50 µl of cold peptide (either long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I at concentrations of 5, 50 and 500 ng) with iodinated long-R^3-IGF-I or long-R^{3}A^{16}-IGF-I (approximately 3 ng, 375 000 c.p.m) overnight at 4° C. The incubation was terminated by the addition of 750 µl of 50 mM Tris, pH 7.4, then centrifuged for 5 min at 4° C followed by resuspension of the pellet in 245 µl of 150 mM NaCl, 50 mM Hepes, pH 7.4. The proteins were cross-linked by the addition of disuccinimidyl suberate dissolved in dimethylsulphoxide in final concentrations of 0.1 mM and 2 % (v/v) respectively, and incubation for 15 min at 4° C. The cross-linking reactions were stopped by the addition of 1 ml of 10 mM Tris, pH 7.4, containing 1 mM EDTA. The reactions were then incubated at room temperature for 5 min, centrifuged for 5 min at 4° C and the supernatants discarded. The pellets were dissolved in 30 µl of 10 mM sodium phosphate buffer, pH 7.0, containing 2 % (w/v) SDS. Aliquots (40 µl) of reducing SDS-load buffer (15 mM Tris, pH 6.8, 10 % (w/v) glycerol, 2 % (w/v) SDS, 4 % (v/v) β-mercaptoethanol, 0.1 % (w/v) bromophenol blue) were added, samples were heat denatured at 65° C for 15 min then analysed by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was based on the procedure of Laemmli (1970), using 0.75 mm gels consisting of a 3 % (w/v, 40 : 1, acrylamide : bis-acrylamide), 0.5 M Tris, pH 6.8, 0.4 % (w/v) SDS stacking gel and a 5 % (w/v, 40 : 1, acrylamide : bis-acrylamide), 1.5 M Tris, pH 8.8, 0.4 % (w/v) SDS separating gel. The proteins were electrophoresed in 0.1 M glycine, 0.1 M Tris, pH 8.3, 0.4 % (w/v) SDS at a constant amperage of 20 mA and 40 mA through the stacker and separating gels, respectively. Following electrophoresis the proteins were detected by Coomassie Brilliant Blue R-250 staining and autoradiography. The gels were stained in 1.25 % (w/v) Coomassie Brilliant Blue R-250, 50 % (v/v) methanol, 10 % (v/v) acetic acid at room temperature overnight then destained in several changes of 50 % (v/v) methanol, 10 % (v/v) acetic acid until the desired level of destaining was achieved. Gels were then soaked for 30 min in Milli-Q water then transferred to Whatman 3 MM paper and dried under vacuum at 65° C for 60 mins. Dried gels were exposed to X-ray film with intensifying screens at -80° C for 2 weeks.

4.3.4 IGF-receptor precipitation and TCA assays

The intactness of the iodinated analogues was estimated by the receptor precipitation and TCA assays. The receptor precipitation assay measured the percentage of radiolabel that bound receptors, as modified from Owens *et al.* (1985) and Read *et al.* (1986) using human placental membrane proteins, prepared as described in Section 4.3.3.1. Briefly, a 50 µl sample was incubated with an excess amount of membrane proteins (320 µg) in 250 µl 0.1 M Tris, pH 7.4, containing 0.5 % (w/v) BSA. After 16 hours incubation at 4° C, 1 ml of chilled 0.01 M Tris, pH 7.2, containing 0.1 M $CaCl_2$ and 0.1 % (w/v) BSA was added, mixed, then incubated for 10 min at 4° C. Samples were centrifuged for 30 min at 4 000 g at 4° C and the supernatants aspirated. Membrane-bound radioactivity was obtained by subtracting residual c.p.m in control blanks (samples without membrane proteins added) from the total c.p.m. bound.

To determine the concentration of membrane receptors required for maximal iodinated peptide binding, human placental membranes (40 to 360 µg protein) were incubated with iodinated analogues (91 pg, 10 000 c.p.m.) in 300 µl 0.1 M Tris, pH 7.4, containing

0.5 % (w/v) BSA using the procedure described above. Membrane bound radioactivity was obtained by subtracting the residual c.p.m. in control blanks from the total c.p.m. bound. Subsequently, 320 µg per 100 µl of membrane receptor proteins were routinely used in the receptor precipitation assays, as at this concentration all the iodinated analogues were maximally bound.

The TCA assay measured the percentage of radiolabel precipitated by TCA using the method of Xian *et al.* (1995). The radioactivity of triplicate aliquot samples (50 µl) was counted in a γ-counter prior to the addition of 700 µl of chilled 0.25 % (w/v) BSA and 200 µl of 50 % (w/v) TCA. After vortexing and incubation on ice for 60 min, samples were centrifuged for 20 min at 4° C. The supernatants were removed and the radioactivity in the TCA-precipitated pellets was measured. The radioactivity in the pellets was expressed as a percentage of the total radioactivity in the samples. The maximum TCA precipitability of the undegraded ^{125}I-labelled analogues averaged 86 %.

The competitive IGF-receptor binding assay was used to assess biological activity of unlabelled peptides. Triplicate 50 µl samples were added to 100 µl of 0.1 M Tris, pH 7.4, containing 0.01 M $CaCl_2$ and 0.5 % (w/v) BSA. A limited amount of membrane proteins (80 µg per 50 µl; isolated as described in Section 4.3.3.1) were added and 100 µl of ^{125}I-labelled long-R^3-IGF-I (183 pg, 20 000 c.p.m.). After 16 hours incubation at 4° C, 1 ml of chilled 0.01 M Tris, pH 7.4, containing 0.1 M $CaCl_2$ and 0.1 % (w/v) BSA was added, mixed then incubated for 10 min. Samples were finally centrifuged for 30 min at 4 000 g at 4° C and the supernatants aspirated. The membrane proteins were then precipitated and the bound radioactivity was measured. The amount of intact peptide was estimated by comparison with a standard curve obtained using unlabelled long-R^3-IGF-I (50 - 50000 pg) as the competing peptide.

4.3.5 Peptide degradation with purified pepsin and subsequent HPLC analysis

Long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I were digested using purified porcine pepsin (enzyme to substrate ratio of 1:10 w/w) in either 10 mM HCl, pH 2.0 or 50 mM glycine,

pH 3.2 in the presence and absence of 154 mM NaCl, followed by analysis on reverse-phase HPLC as described in Section 2.3.1.

4.4 RESULTS

4.4.1 Assay development

This section describes the establishment of the digestion and assay conditions which were used to assess the stability of the iodinated and non-iodinated IGF-I analogues.

4.4.1.1 Assay conditions

The concentration of membrane proteins required in the receptor precipitation assay

The placental membrane preparation was estimated as having a protein content of 8.13 mg/ml, using the modified Lowry method (Section 4.3.3.2). Binding curves for the iodinated analogues to human placental membranes were then determined to measure the concentration of human placental membranes required for maximal binding of the iodinated analogues (Figure 4.1). The radiolabelled analogues (91 pg, 10 000 c.p.m.) were incubated with placental membranes at concentrations ranging from 40 to 360 µg/100 µl of placental protein, and the percentage of bound radiolabelled analogue determined. The binding curves showed long-R^3-IGF-I, long-R^3A^{16}-IGF-I, long-R^3E^{53}-IGF-I and long-R^3Q^{59}-IGF-I bound similarly to the receptors with a maximum of 37.5 % of the added radioligand binding to the membranes when concentrations exceeded 280 µg/100 µl of placental protein (Figure 4.1). Long-R^3L^{25}-IGF-I and long-R^3V^{10}-IGF-I showed reduced binding to the receptor membranes, giving maximal binding values of 22 % and 6 % respectively of the added radioactivity at membrane concentrations greater than 280 µg/100 µl (Figure 4.1). Therefore, when determining the amount of intact iodinated analogue binding to placental membranes in the receptor precipitation assay, 320 µg/100 µl of placental protein was routinely used in the analysis. Due to the limited receptor binding of long-R^3V^{10}-IGF-I, its stabilities could only be measured by TCA precipitability.

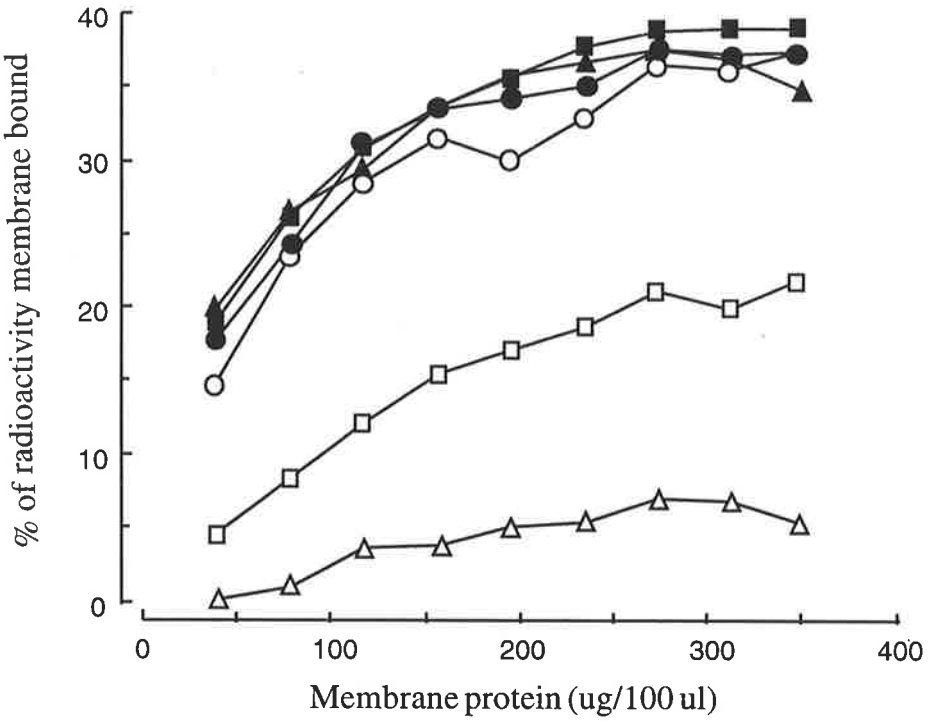


FIGURE 4.1
Binding of iodinated long-R^3-IGF-I analogues to human placental membrane proteins.

The proteins tested were:

- ● long-R^3-IGF-I;
- ○ long-R^{3}A^{16}-IGF-I;
- ■ long-R^{3}E^{53}-IGF-I;
- □ long-R^{3}L^{25}-IGF-I;
- ▲ long-R^{3}Q^{59}-IGF-I;
- △ long-R^{3}V^{10}-IGF-I.

Values are the means of triplicate determinations at each peptide concentration. SEM values are less than 5 %.

Comparison of standard curves used to assess intact unlabelled analogues

When comparing the degradation rates of non-labelled long-R^3-IGF-I and long-R^3A^{16}-IGF-I, the amount of intact peptide is estimated by comparison with a standard curve. This curve is obtained by measuring the binding of a radiolabelled peptide to placental membranes in the presence of competing and increasing concentrations of unlabelled peptide. A reference sample contains radiolabelled peptide in the absence of a competing unlabelled ligand. The aim of this experiment was to determine if differences occurred between standard curves when using either long-R^3-IGF-I or long-R^3A^{16}-IGF-I as the radiolabelled or competing peptide.

Standard curves obtained when using ^{125}I-labelled long-R^3-IGF-I with either long-R^3-IGF-I or long-R^3A^{16}-IGF-I as the competing non-labelled peptide were very similar, as shown in Figure 4.2a. The use of either ^{125}I-labelled long-R^3-IGF-I or long-R^3A^{16}-IGF-I with the same competing non-labelled peptide also resulted in similar curves which gave comparable concentrations of unlabelled peptide at half maximal binding of added radioligand (Figure 4.2b).

It was also important to determine the effect of stomach flushings on the standard curves. As shown by Figure 4.2c, the addition of flushings had a minor effect. Therefore, when estimating the amount of intact unlabelled peptide by comparison with a standard curve, the standard curves were routinely determined using long-R^3-IGF-I as both the radiolabelled and competing peptide. When samples contained stomach flushings, an equivalent amount of stomach flushings were also added to the samples comprising the standard curve.

Cross-linking of membrane receptor

Long-R^3-IGF-I and long-R^3A^{16}-IGF-I were cross-linked to the human placental membrane preparation to confirm that the peptides were binding to receptors present on the membranes. The radiolabelled peptides were cross-linked to the placental membranes then electrophoresed in SDS under reducing conditions followed by autoradiography to measure the molecular weight of the receptor-ligand complex, as shown in Figure 4.3. A band

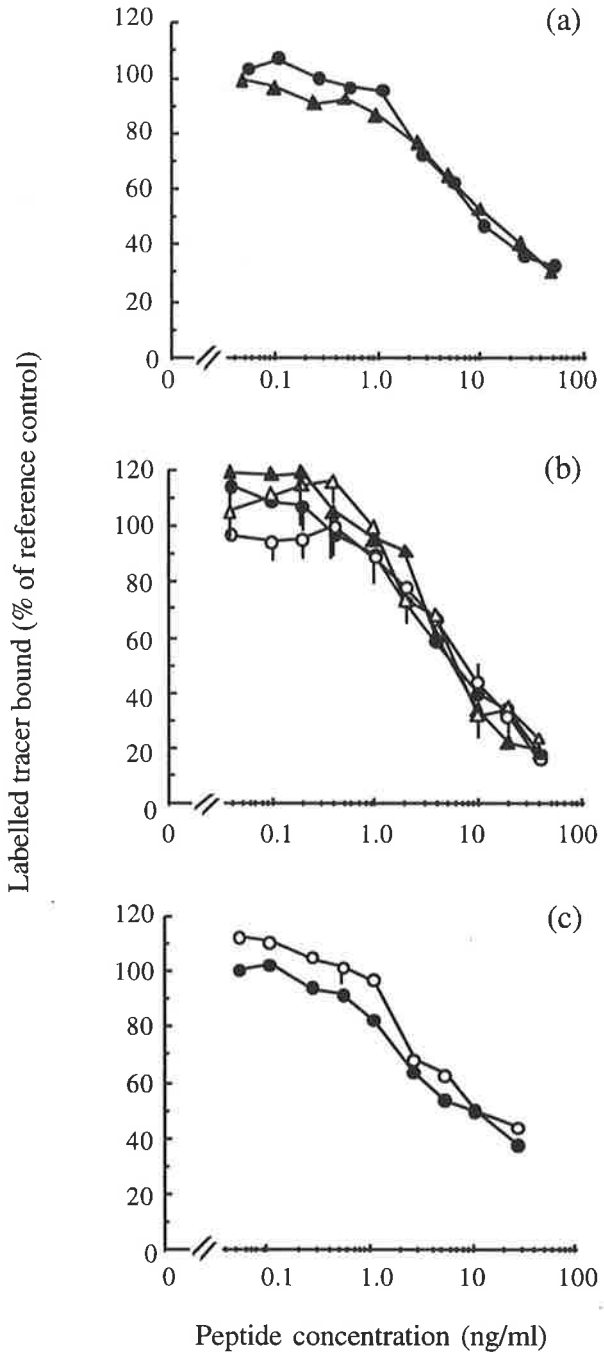


FIGURE 4.2

Comparisons of standard curves for assessing unlabelled peptide.

(a) Comparison of ^{125}I-labelled long-R^3-IGF-I binding to human placental membranes in the presence of competing unlabelled peptide, long-R^3-IGF-I (●) or long-R^{3}A^{16}-IGF-I (▲).

(b) Competition of ^{125}I-labelled long-R^3-IGF-I (circles) and ^{125}I-labelled long-A^{16}-R^3-IGF-I (triangles) for binding to human placental membranes in the presence of competing unlabelled long-R^3-IGF-I (filled symbols) or long-R^{3}A^{16}-IGF-I (open symbols).

(c) Comparison of ^{125}I-labelled long-R^3-IGF-I binding to human placental membranes, using unlabelled long-R^3-IGF-I as the competing peptide, in the presence (●) and absence (○) of rat stomach flushings.

Vaules are the means of triplicate determinations at each peptide concentration. SEM vaules greater than 5 % are shown.

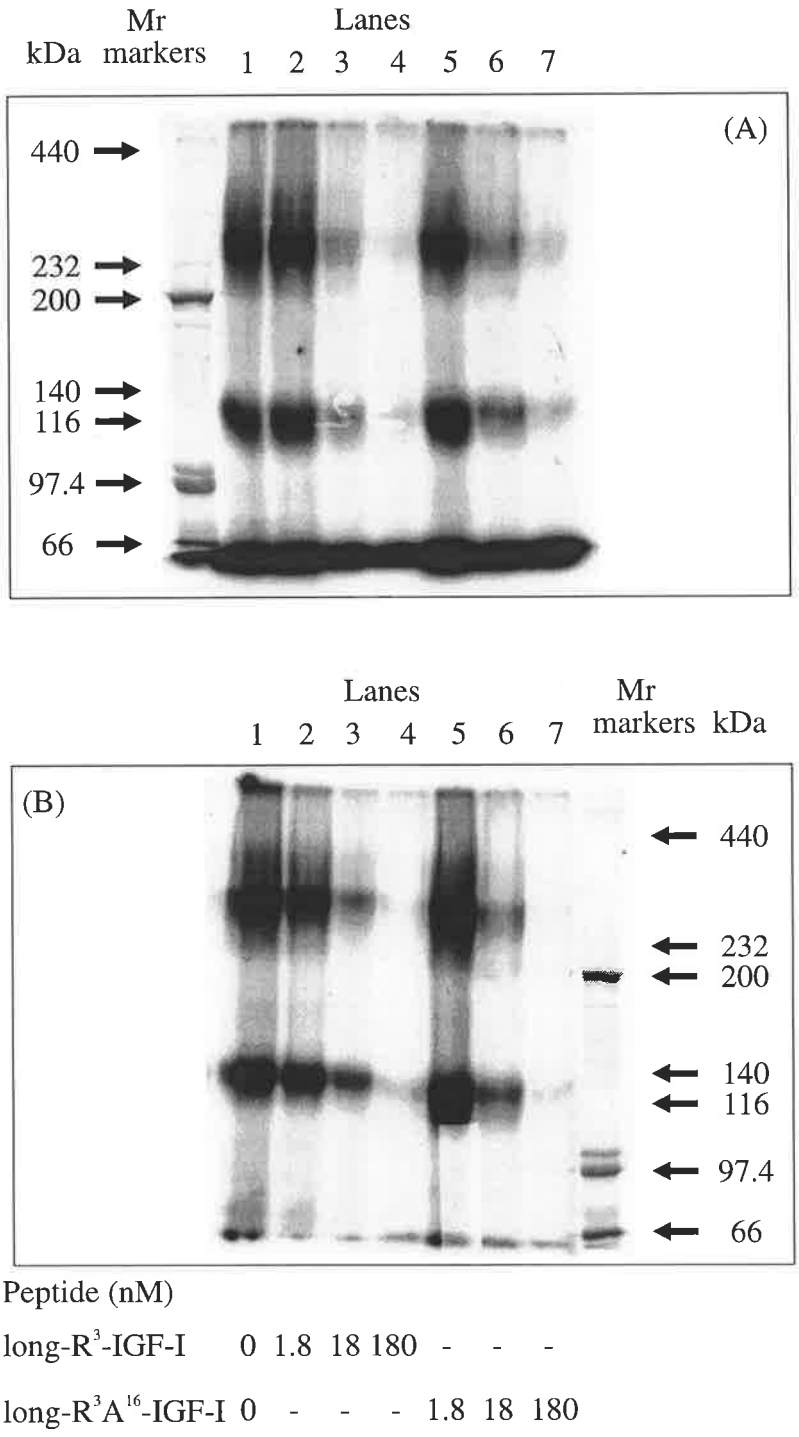


FIGURE 4.3
Autoradiographs of cross-linked ^{125}I-labelled long-R^3-IGF-I and ^{125}I-labelled long-R^{3}A^{16}-IGF-I to human placental membrane proteins.
Labelled long-R^3-IGF-I (gel A) and labelled long-R^{3}A^{16}-IGF-I (gel B) were cross-linked to membrane proteins in the presence of increasing concentrations of unlabelled peptides then electrophoresed on SDS-polyacrylamide gels under reducing conditions. The concentrations of unlabelled peptides were: lane 1, no addition; lanes 2, 3 and 4, addition of 1.8 nM, 18 nM and 180 nM of long-R^3-IGF-I, respectively; lanes 5, 6, and 7, addition of 1.8 nM,18 nM and 180 nM of long-R^{3}A^{16}-IGF-I, respectively. Relative molecular mass of the standard protein markers are shown in kDa and are indicated by arrows.

corresponding to a relative molecular mass of 130 000 was detected in the autoradiographs. This band is similar to that described in other tissues for the α subunit of the type 1 IGF-receptor (Arafah, 1991; Chernausek *et al.*, 1987: Bhaumick & Bala, 1987). The intensity of the band was decreased when increasing amounts of unlabelled long-R^3-IGF-I and long-R^3A^{16}-IGF-I (1.8, 18 and 180 nM) were added during the binding experiment (Figure 4.3, lanes 2 -7). An additional band with a molecular weight of approximately 270 000 Da was also noted, and the intensity of this band similarly decreased with increasing amounts of unlabelled peptide. As chemical cross-linking can affect aggregation of cross-linked molecules, this band could represent the intact receptor or alternatively, polymerisation of the α subunit (Arafah, 1991; Chernausek *et al.*, 1987: Bhaumick *et al.*, 1987). This band is unlikely to represent binding of labelled long-R^3-IGF-I or long-R^3A^{16}-IGF-I to the type 2 receptor, since IGF-I analogues have a markedly reduced affinity for this receptor (Francis *et al.*, 1993).

4.4.1.2 Digestion conditions

An initial experiment with rat stomach flushings showed *in vitro* degradation of long-R^3-IGF-I and the analogues was occurring in the time zero control compared to a control containing no stomach flushings. In the TCA precipitation assay for example, the difference between the two controls ranged from 25.3 % for long-R^3A^{16}-IGF-I to 48.1 % for long-R^3V^{10}-IGF-I, and even greater differences were observed in the receptor precipitation assay. This effect was presumed to be due to degradation of the peptides in the time zero control by contaminating duodenal enzymes present in the stomach flushings. Duodenal enzymes, such as trypsin and chymotrypsin, would not be inhibited in the presence of the stopping buffer, 100 mM Tris, 154 mM NaCl, pH 8.0, unlike pepsin which is irreversibly denatured above pH 6.0 (Fruton, 1971). Therefore, the stability of long-R^3-IGF-I under a variety of conditions using both stomach and duodenal flushings was investigated, to determine digestion conditions where degradation of the long-R^3-IGF-I analogues could be attributed to pepsin activity alone. Degradation samples were analysing using TCA precipitation (Figure 4.4). When duodenal flushings were treated under the same protocol

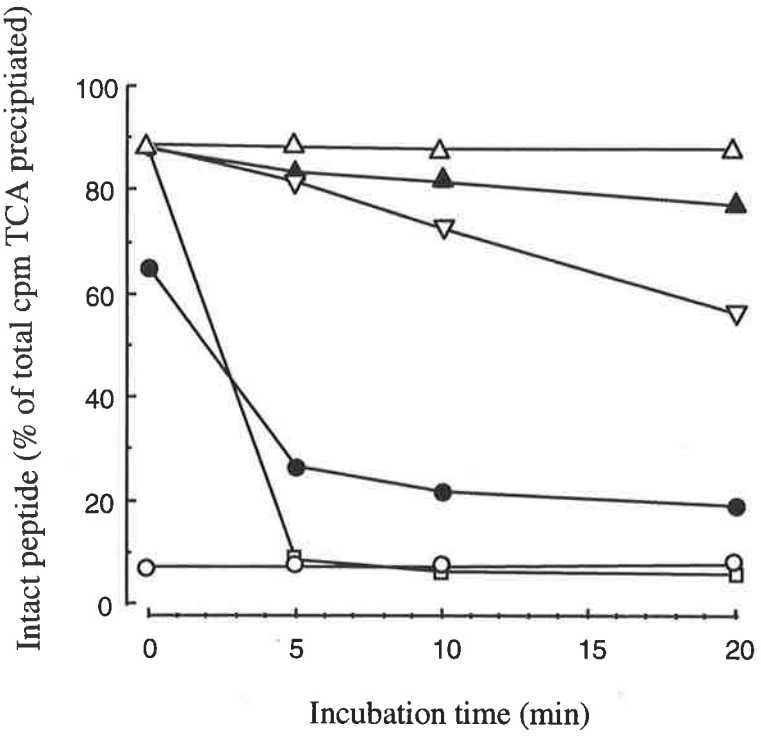


FIGURE 4.4

***In vitro* degradation of long-R^3-IGF-I in luminal flushings with different digestion and stopping conditions.**

Aliquots of stomach (filled symbols) or duodenal (open symbols) flushings were incubated with radiolabelled long-R^3-IGF-I for different periods of time under the following conditions:

	DIGESTION BUFFER	STOPPING BUFFER
●	pH 3.2	pH 8.0
▲	pH 3.2 + inhibitors	pH 8.0
□	154 mM NaCl	pH 4.0
▽	pH 3.2	pH 4.0
○	pH 3.2	pH 8.0
△	pH 3.2 + inhibitors	pH 4.0

Where inhibitors are 1 mM PMSF and 0.05 % (w/v) aprotinin; pH 3.2 digestion buffer is 50 mM glycine, pH 3.2, 154 mM NaCl; pH 8.0 stopping buffer is 0.1 M Tris, pH 8.0, 154 mM NaCl; and pH 4.0 stopping buffer is 0.1 M sodium acetate, pH 4.0, 1 mM PMSF, 0.05 % (w/v) aprotinin. Percentage of intact peptide was estimated by TCA precipitation. Values are the means of triplicate determinations at each time-point with SEM values of less than 5 %.

used for stomach flushings (digestion buffer: 50 mM glycine buffer, pH 3.2, 154 mM NaCl; stopping buffer: 0.1 M Tris, pH 8.0, 154 mM NaCl), a marked degradation occurred in the time zero control in comparison to the control containing no flushings. Although the rates were different, the duodenal flushings were also shown to degrade long-R^3-IGF-I in 10 mM Tris, pH 7.4, and 50 mM glycine, pH 3.2. The addition of the protease inhibitors, PMSF and aprotinin, to the duodenal flushings in 50 mM glycine, pH 3.2, prevented peptide degradation. Therefore, 0.05 % (w/v) aprotinin and 1 mM PMSF were routinely added to degradation experiments using stomach flushings to prevent peptide degradation due to duodenal enzymes. Under these conditions, a variation of less than 1 % was observed between the time zero and no flushing control. This subsequently allowed an assessment of pepsin activity on the added peptides.

4.4.2 Stability of iodinated IGF analogues in rat luminal flushings

The stabilities of the complete range of analogues, long-R^3V^{10}-IGF-I, long-R^3A^{16}-IGF-I, long-R^3L^{25}-IGF-I, long-R^3E^{53}-IGF-I, long-R^3Q^{59}-IGF-I and long-R^3-IGF-I, were assessed in gut flushings. As limited amounts of the analogues were available, iodinated peptides were used. The *in vitro* degradation of the analogues was performed three times using independent pools of stomach flushings. Iodinated long-R^3A^{16}-IGF-I showed the greatest stability with a half-life of 7.0 ± 2.0 min (mean ± SEM) compared to 4.2 ± 1.5 min for long-R^3-IGF-I. Long-R^3Q^{59}-IGF-I showed an approximate equivalent stability to long-R^3-IGF-I, with long-R^3E^{53}-IGF-I indicating a slightly decreased stability and long-R^3L^{25}-IGF-I a slight increase (Figure 4.5a).

Degradation of the ^{125}I labelled analogues was also analysed by TCA precipitation. By this criterion, degradation was considerably slower, suggesting incomplete degradation by pepsin to residual large peptide fragments (Figure 4.5b). The stability trend noted in the receptor precipitation assay was similarly shown using TCA precipitation analysis.

In addition to assessing the stability of the long-R^3-IGF-I analogues in rat stomach flushings, the stability of these peptides was also determined in rat duodenal flushings using the TCA precipitation method (Figure 4.6). Degradation to TCA-soluble fragments was rapid

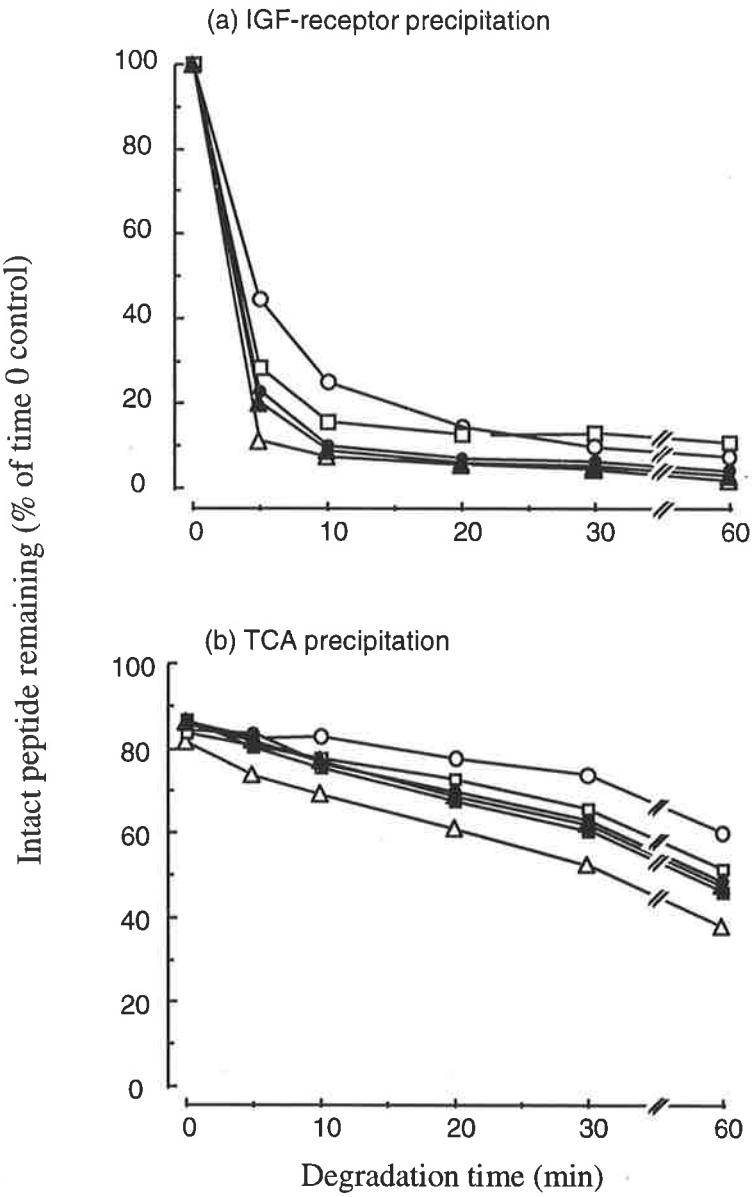


FIGURE 4.5

Degradation of ^{125}I-labelled long-R^3-IGF-I analogues in rat stomach flushings.

Aliquots of flushings were incubated with radiolabelled long-R^3-IGF-I analogues for different periods of time, and the percentage of intact analogue was estimated by (a) IGF-receptor precipitation and (b) TCA precipitation. Values are means of triplicate determinations at each time point and are expressed as a % of the time zero control. SEM values are less than 5 %. The symbols used are: ● long-R^3-IGF-I; ○ long-R^{3}A^{16}-IGF-I; ■ long-R^{3}E^{53}-IGF-I; □ long-R^{3}L^{25}-IGF-I; ▲ long-R^{3}Q^{59}-IGF-I; △ long-R^{3}V^{10}-IGF-I.

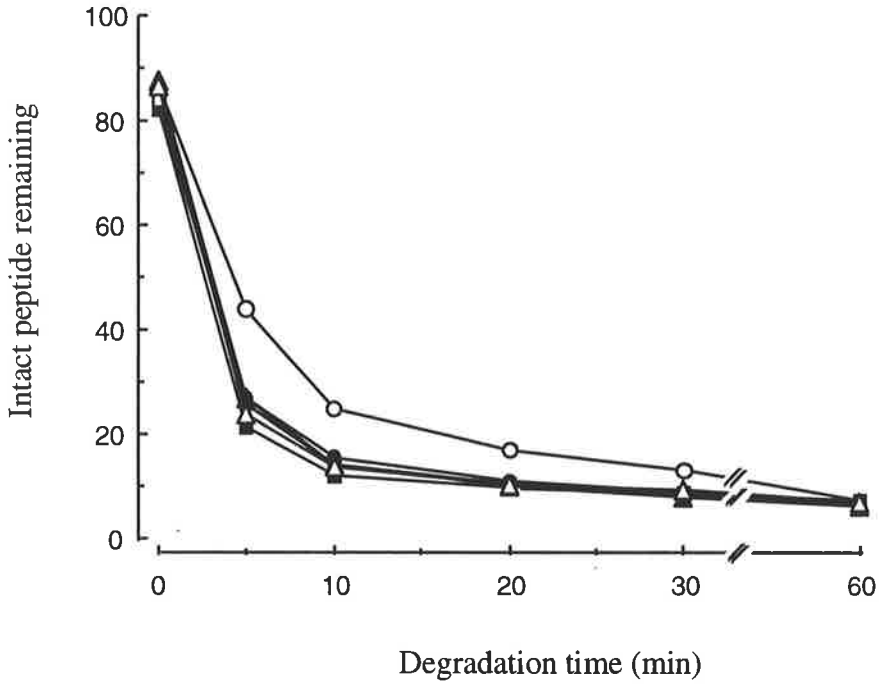


FIGURE 4.6
Degradation of ^{125}I-labelled long-R^3-IGF-I analogues in rat duodenal flushings.
Aliquots of flushings were incubated with radiolabelled long-R^3-IGF-I analogues for different periods of time, and the percentage of intact analogue was estimated by TCA precipitation. Values are means of triplicate determinations at each time point and are expressed as a % of the time zero control. SEM values are less than 5 %. The symbols used are: ● long-R^3-IGF-I; ○ long-R^{3}A^{16}-IGF-I; ■ long-R^{3}E^{53}-IGF-I; □ long-R^{3}L^{25}-IGF-I; ▲ long-R^{3}Q^{59}-IGF-I; and △ long-R^{3}V^{10}-IGF-I.

for all the analogues giving half-lives of less than 5 min. Nevertheless, long-R^3A^{16}-IGF-I showed a slightly increased half-life (3.9 ± 0.7 min) compared to long-R^3-IGF-I and the other analogues (2.3 ± 0.3 min). This increased stability of long-R^3A^{16}-IGF-I in duodenal flushings is consistent with the Phe16Ala mutation removing a putative chymotrypsin cleavage site.

4.4.3 Stability of nonlabelled IGF analogues in rat stomach flushings

As long-R^3A^{16}-IGF-I showed the most enhanced pepsin stability of the long-R^3-IGF-I analogues prepared, further experiments involved only comparisons between this peptide and the parent material, long-R^3-IGF-I.

To determine the possibility that iodination modified the relative stability of the peptides, the degradation rates of non-labelled long-R^3-IGF-I and long-R^3A^{16}-IGF-I were measured in rat stomach flushings. Stability was assessed using a competitive IGF-receptor binding assay in order to estimate retention of biological activity.

The degradation of non-labelled long-R^3-IGF-I and long-R^3A^{16}-IGF-I was performed four times using independent pools of stomach flushings. Similar to the results with iodinated peptides, the long-R^3A^{16}-IGF-I analogue was approximately 2-fold more stable than long-R^3-IGF-I, with respective half-lives of 11.25 ± 2.82 min (mean ± SEM) and 5.65 ± 1.33 min (Figure 4.7). Addition of pepstatin, an aspartic protease inhibitor, to the digestion reaction, almost completely inhibited the degradation of the peptides (Figure 4.7), indicating that peptide degradation in the stomach flushing was most likely due to pepsin activity.

4.4.4 The effect of stomach flushings from different species on peptide stability

The degradation rates of long-R^3-IGF-I and long-R^3A^{16}-IGF-I were measured in rat, human, and pig stomach flushings and compared to that obtained with purified porcine pepsin. These experiments were performed to determine if the stability of the IGF analogues differed between species.

Initially the pepsin activity in the stomach flushings from different species were standardised against a substrate unrelated to IGF-I. Denatured haemoglobin was used as the substrate to determine the total pepsin activity of the flushings. The pepsin activity against

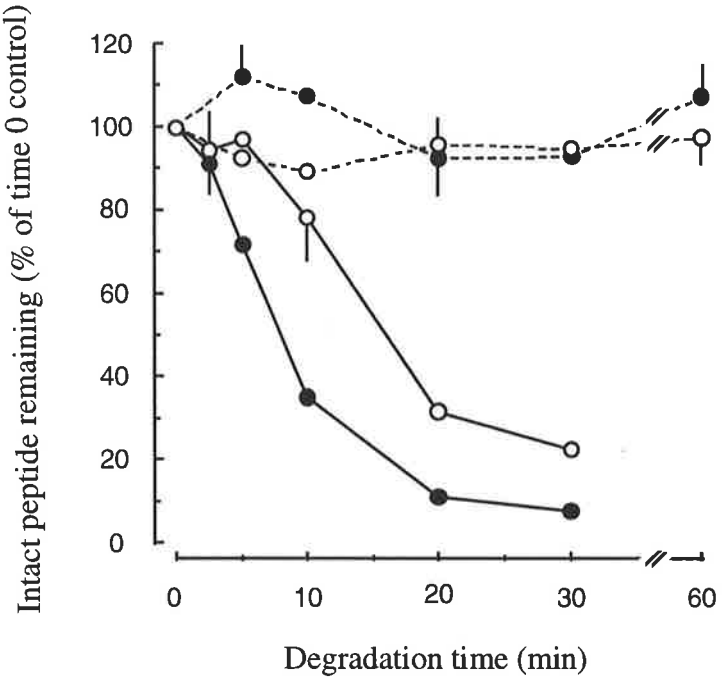


FIGURE 4.7
Degradation of unlabelled long-R^3-IGF-I and long-R^3A^{16}-IGF-I in rat stomach flushings and the effect of pepstatin.
Aliquots of flushings were incubated with long-R^3-IGF-I (●) and long-R^3A^{16}-IGF-I (o) for different time periods. Samples were assayed using a competitive IGF-receptor binding assay to give estimates of intact peptides remaining. Values are means of triplicate determinations at each time point and are expressed as a % of the time zero control. SEM values greater than 5 % are indicated by bars. The presence of 1 M pepstatin in the incubation mixture is indicated by the dotted line.

haemoglobin in the rat flushing (1.65 mg/ml) was similar to that in the human flushing (1.9 mg/ml), although it was 10-fold higher than in the pig (0.16 mg/ml).

On the basis of these measurements, the stabilities of long-R^3-IGF-I and long-R^3A^{16}-IGF-I were tested against purified porcine pepsin and stomach flushings, standardised to pepsin activities of 160 µg/ml (Figure 4.8). Long-R^3-IGF-I (Figure 4.8a) was most stable in rat stomach flushings with a half-life of 23 min. Human and pig flushings rapidly degraded long-R^3-IGF-I, giving half-lives of less than 15 min in both cases. Purified porcine pepsin, standardised for activity against haemoglobin, degraded long-R^3-IGF-I slightly more rapidly than an equivalent activity in pig stomach flushings. A similar degradation pattern was obtained with long-R^3A^{16}-IGF-I where half-lives of 43, 12 and 9 min were measured with rat, human and porcine flushings respectively and less than 5 min with purified porcine pepsin (Figure 4.8b).

To determine accurately the relative stability of long-R^3-IGF-I and long-R^3A^{16}-IGF-I, the stomach flushings were diluted with glycine buffer to obtain half-lives of greater than 20 min. To achieve this, the human and porcine flushings were diluted 10-fold and the rat flushings, 1.5-fold. Long-R^3A^{16}-IGF-I had greater stability than long-R^3-IGF-I in flushings from all of the species examined (Table 4.1). The differential in stability between long-R^3A^{16}-IGF-I and long-R^3-IGF-I was slightly decreased in human flushings (1.7-fold), compared to rat and pig stomach flushings (2.1-fold).

In summary, stomach flushings of the rat, human and pig, standardised for proteolytic activity against haemoglobin substrate, varied considerably in their ability to degrade IGF-I analogues. Nevertheless, the mutated analogue long-R^3A^{16}-IGF-I proved more stable than the parent molecule in all cases.

4.4.5 The effect of buffer and salt on peptide digestion

From the results shown above, both iodinated and unlabelled long-R^3A^{16}-IGF-I were approximately 2-fold more stable than the parent material when assayed *in vitro* with stomach flushings. In comparison, the reverse-phase HPLC analysis of pepsin digests showed long-R^3A^{16}-IGF-I had an enhanced stability of approximately 10-fold compared to

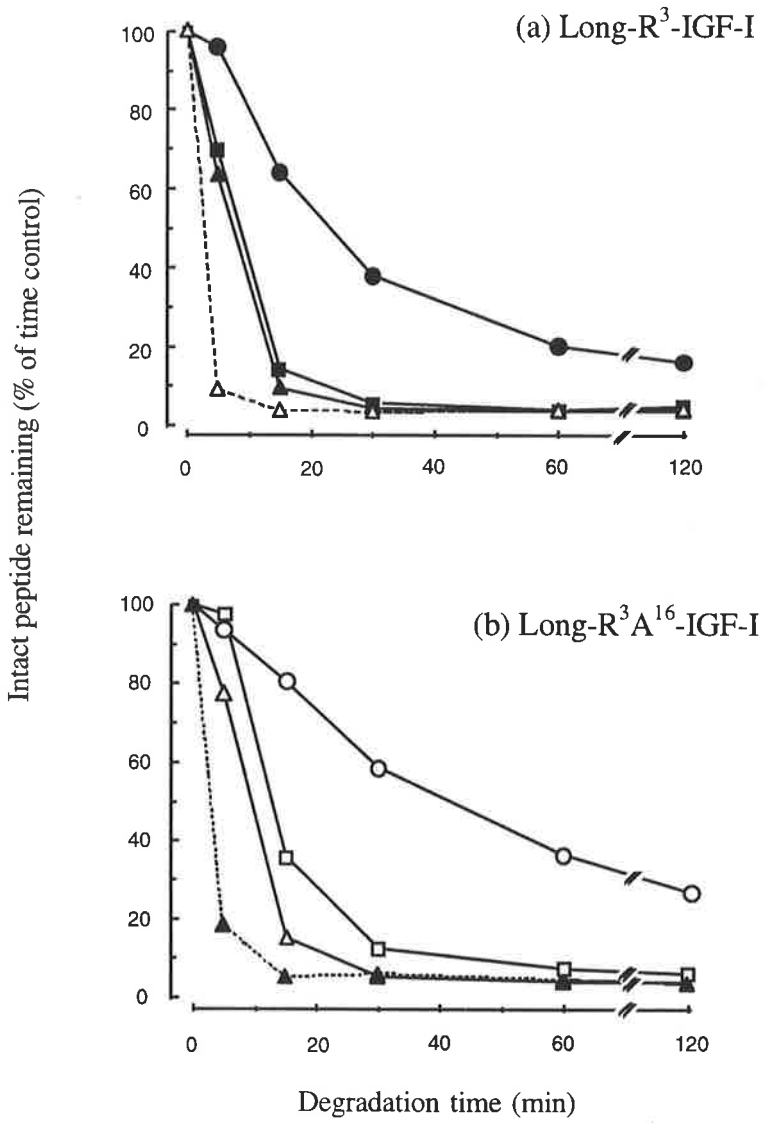


FIGURE 4.8

Comparison of the degradation rates of long-R^3-IGF-I and long-R^3A^{16}-IGF-I in stomach flushings from various species and purified porcine pepsin.

Aliquots of purified pepsin (dotted line), or rat (circles), human (squares) and pig (triangles) stomach flushings, standardised to pepsin activities of 160 g/ml on denatured haemoglobin, were incubated with (a) radiolabelled long-R^3-IGF-I or (b) radiolabelled long-R^3A^{16}-IGF-I for different periods of time. The percentage of intact long-R^3-IGF-I in each sample was estimated by IGF-receptor precipitation. The values are means of triplicate determinations at each time point and are expressed as a % of the time zero control. SEM values are less than 5 %.

TABLE 4.1
Comparison of ^{125}I-labelled long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I degradation rates in stomach flushings from several species, and purified porcine pepsin.

	Pepsin activity in assay † (μg/ml)	long-R^3-IGF-I* half life (min)	long-R^{3}A^{16}-IGF-I* half life (min)	Relative‡ difference (fold)
Pig stomach flushings	12.8	17	36	2.1
Human stomach flushings	13.7	45	75	1.7
Rat stomach flushings	96.4	22	46	2.1
Purified porcine pepsin	7.0	30	80	2.7

† Pepsin activity was determined by using denatured haemoglobin as a substrate.
* Half lives were obtained using an IGF-receptor binding precipitation assay with human placental membranes.
‡ Ratio of half-life of long-R^{3}A^{16}-IGF-I to that of long-R^3-IGF-I.

long-R^3-IGF-I (Chapter 3). To reconcile these apparent conflicting results, a competitive IGF-receptor assay was used to assess peptide integrity following pepsin digestion under the conditions described in Chapter 3, namely unlabelled peptide digestion in 10 mM HCl, pH 2.0.

As shown in Figure 4.9, the level of receptor-binding long-R^3-IGF-I peptide decreased rapidly over the 90 min digestion period. In comparison, long-R^3A^{16}-IGF-I was much more stable and initially even showed an apparently increased binding, followed by a slow decline in binding with time (Figure 4.9). The initial increase in binding is presumably due to the rapid cleavage of the *N*-terminal extension peptide, forming the FVN-R^3-IGF-I analogue which has a 2-fold higher affinity for the type 1 IGF-receptor than the parent molecule (Section 2.4.2). Although the parent molecule, long-R^3-IGF-I, has an identical *N*-terminal extension, which is also rapidly cleaved by pepsin, no enhancement in receptor binding was seen during incubation with pepsin. Because of the high stability of the long-R^3A^{16}-IGF-I analogue, the time to achieve a 25 % loss of binding was used as the measure of stability. This required 80 min for long-R^3A^{16}-IGF-I, in comparison with 3 min for long-R^3-IGF-I (Figure 4.9), representing an increase in stability of the analogue by approximately 25-fold. The assay was repeated, using a different aliquot of pepsin, and between the two experiments an average half-life of 6.5 ± 3 min and 67 ± 14 min was measured for long-R^3-IGF-I and long-R^3A^{16}-IGF-I, respectively.

These results suggested that the conditions of the degradation were important determinants of the relative pepsin stability of long-R^3-IGF-I and long-R^3A^{16}-IGF-I. Since the stomach environment also changes in association with differences in concentrations of food components, acidity and salt, it can be expected that IGF-I would show similar variation in stability *in vivo*. Consequently, the effect of salt and pH on the rate of pepsin digestion of long-R^3-IGF-I and long-R^3A^{16}-IGF-I was examined more closely.

The effects of changes in pH on degradation rates of long-R^3-IGF-I and long-R^3A^{16}-IGF-I were determined by simultaneously comparing the buffers, 10 mM HCl, pH 2, and 50 mM glycine, pH 3.2, at an enzyme : substrate ratio of 1 : 2.5 (w/w). The stability of the non-iodinated analogues was assessed using a competitive IGF-receptor

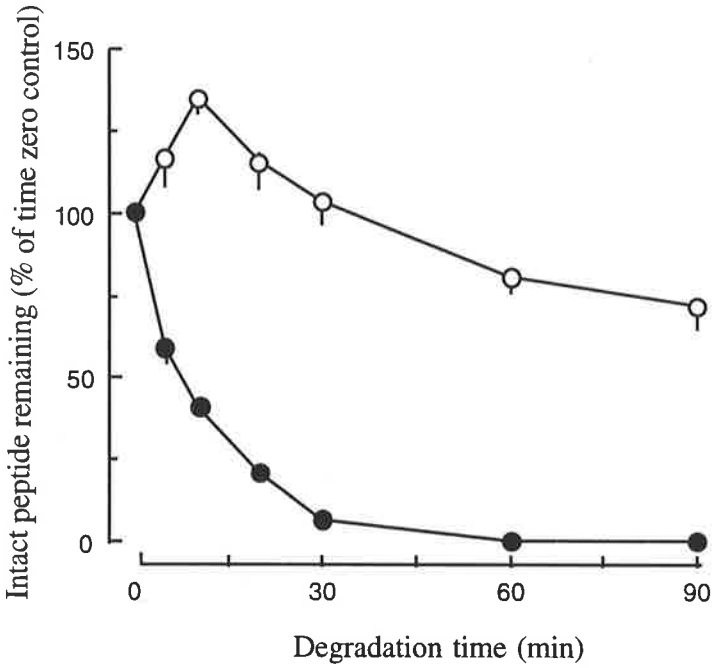


FIGURE 4.9

Comparison of the membrane receptor binding of long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I after pepsin digestion.

Long-R^3-IGF-I and long-R^{3}A^{16}-IGF-I were digested with pepsin at an enzyme : substrate ratio of 20 : 1 at 37° C. Samples, taken at various time points, were assayed using a competitive IGF-receptor binding assay to give an estimate of intact peptide remaining. Values are means of triplicate determinations at each time-point, with SEM values indicated by the descending bars where they are larger than the symbols.

Symbols used are: ● , long-R^3-IGF-I; ○ , long-R^{3}A^{16}-IGF-I.

binding assay. Both long-R^3-IGF-I and long-R^3A^{16}-IGF-I were at least 10-fold more stable in HCl than in glycine (Figure 4.10). The half-lives of long-R^3-IGF-I and long-R^3A^{16}-IGF-I were 48 min and greater than 120 min respectively in HCl, compared to less than 5 min and 10 min in glycine. As noted previously, the long-R^3A^{16}-IGF-I peptide showed an initial increase in receptor binding activity in the HCl buffer. This increased receptor binding activity was not apparent in the glycine buffer, although the rapid degradation may have masked a response in the initial few minutes of degradation.

To determine the effect of salt on degradation rate, the above experiments were repeated in the presence of 154 mM NaCl. Both growth factors were less stable in the presence of saline (Figure 4.11). In HCl, the addition of saline reduced the half-life of long-R^3-IGF-I from 48 min to 10 min. The differential in stability was even greater for long-R^3A^{16}-IGF-I, where the half-life fell from over 120 min in HCl to only 14 min in HCl/saline. Similar, although less pronounced effects of saline were apparent in glycine buffer where the half-life of long-R^3A^{16}-IGF-I was measured at 10 and 8 min, respectively, in glycine and glycine/saline (full time-course not shown).

In summary, the peptides were most stable at the lower pH in the absence of NaCl. Increasing the pH to 3.2, or the addition of NaCl decreased stability of both peptides, but in particular that of the mutant analogue, long-R^3A^{16}-IGF-I. These results were also confirmed by reverse-phase HPLC analysis under similar buffer conditions.

The results of the HPLC analyses of long-R^3A^{16}-IGF-I degradation, after 15 min at 37° C using an enzyme to substrate ratio of 1 : 10 (w/w), are shown in Figure 4.12. After 15 min pepsin digestion, no intact long-R^3A^{16}-IGF-I peptide was present under any of the buffer conditions examined: 10 mM HCl, 10 mM HCl + 154 mM NaCl, 50 mM glycine, pH 3.2, and 50 mM glycine, pH 3.2, + 154 mM NaCl. Assessment of pepsin stability, as shown by the presence of the initial digestion fragment, FVN-R^3A^{16}-IGF-I, showed stability was markedly enhanced in 10 mM HCl compared with the other digestion buffers. The presence of saline in both the HCl or glycine buffers resulted in enhanced digestion of the FVN-R^3A^{16}-IGF-I peptide as shown by a reduction in the peptide fragment peak and a subsequent increase in the number and size of peptide fragments eluting in lower acetonitrile

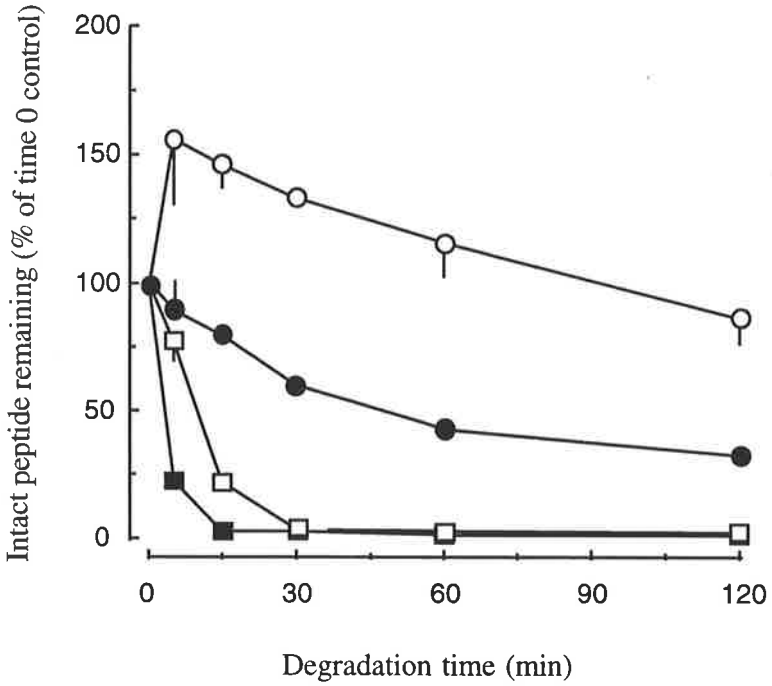


FIGURE 4.10
Comparison of the degradation rates of long-R^3-IGF-I and long-R^3A^{16}-IGF-I with purified porcine pepsin in HCl, pH 2.0 and glycine buffer, pH 3.2.
Peptides were incubated with pepsin at 37° C with an enzyme : substrate ratio of 1 : 2.5. At various time points samples were taken and assayed using a competitive IGF-receptor binding assay. Values are means of triplicate determinations at each time point and are expressed as a % of the time zero control. SEM values of greater than 5 % are indicated by bars. Closed symbols represent long-R^3-IGF-I and open symbols, long-R^3A^{16}-IGF-I, with circles denoting 10 mM HCl, pH 2.0, and squares, 50 mM glycine, pH 3.2.

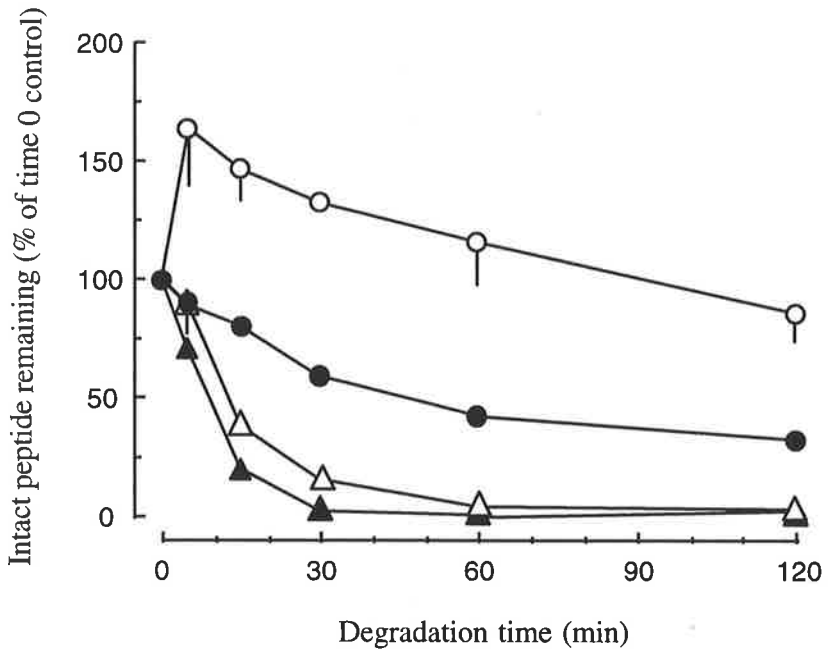


FIGURE 4.11
The effect of NaCl on degradation rates of long-R^3-IGF-I and long-R^3A^{16}-IGF-I using purified porcine pepsin in HCl, pH 2.0.
Long-R^3-IGF-I (closed symbols) and long-R^3A^{16}-IGF-I (open symbols) were incubated with pepsin in 10 mM HCl, pH 2.0, at 37° C in the presence (triangles) and absence (circles) of 154 mM NaCl. Samples, taken at various time points, were assayed by an IGF-receptor competitive binding assay. Values are means of triplicate determinations at each time point and are expressed as a % of the time zero control. Descending bars indicate SEM values greater than 5 %.

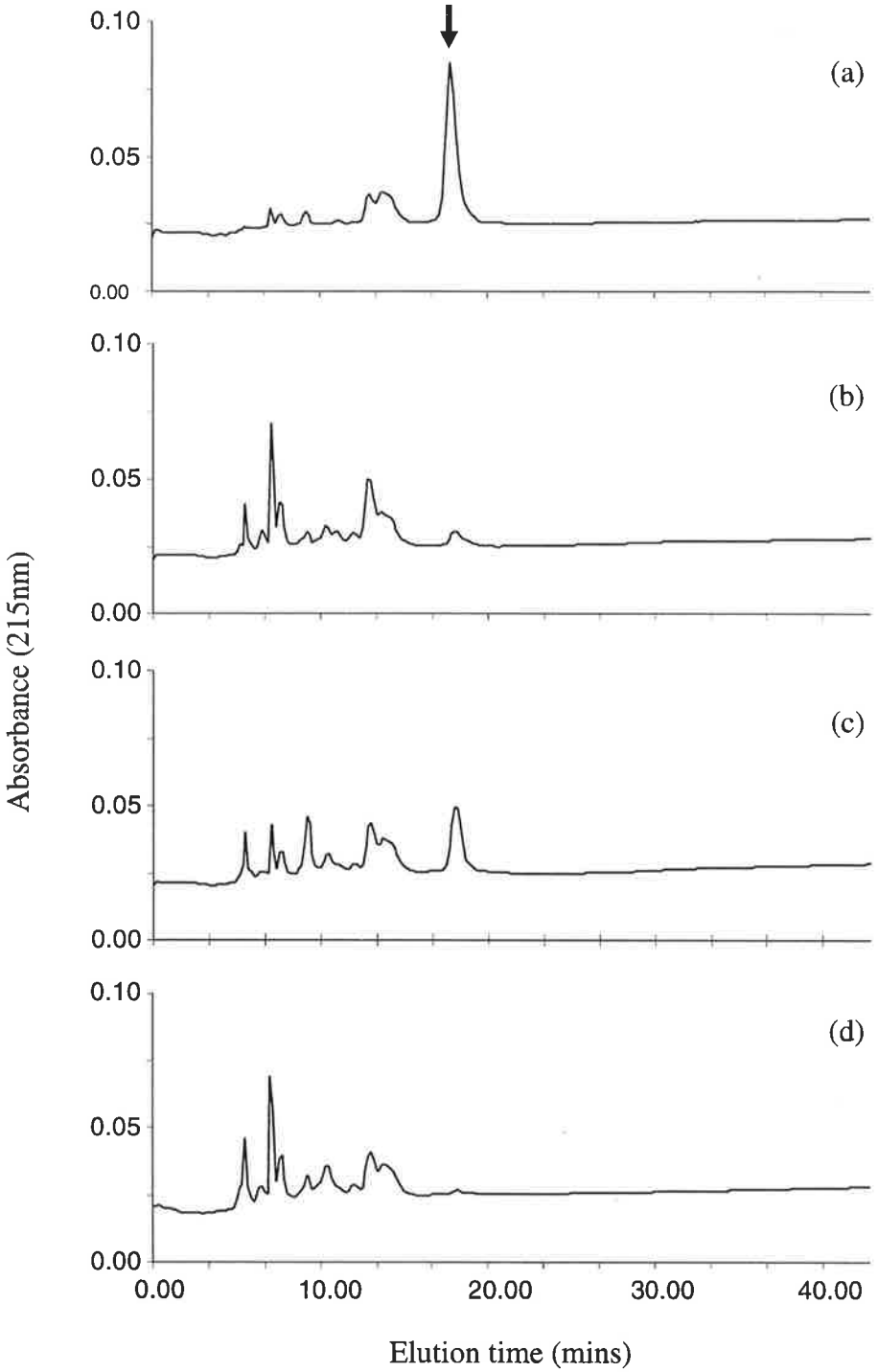


FIGURE 4.12
Comparison of the pepsin digestion of long-R^3A^{16}-IGF-I in different digestion buffers, as analysed by reverse-phase HPLC.
Digestions were performed in (a) 10 mM HCl, pH 2.0, (b) 10 mM HCl, pH 2.0 + 154 mM NaCl, (c) 50 mM glycine, pH 3.2, (d) 50 mM glycine, pH 3.2 + 154 mM NaCl using an enzyme : substrate ratio of 1 : 10 at 37° C. The 15 min time-point was analysed by HPLC on a Brownlee Aquapore C4 column using a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at 215 nm. Intact long-R^3A^{16}-IGF-I elutes at approximately 27 min under these conditions. The digestion fragment, FVN-R^3A^{16}-IGF-I, is indicated by the arrow.

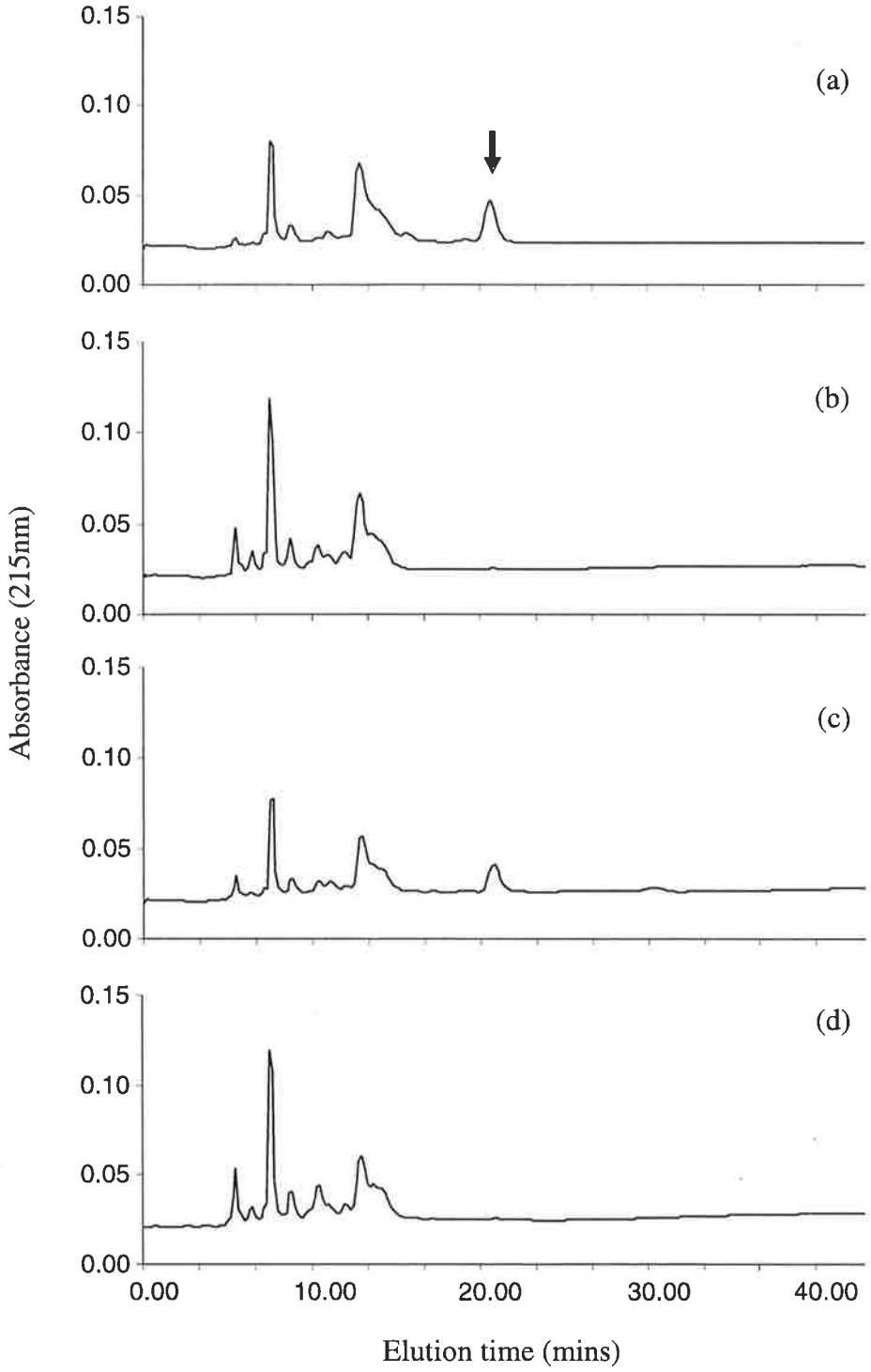


FIGURE 4.13
Comparison of the pepsin digestion of long-R^3-IGF-I in different digestion buffers, as analysed by reverse-phase HPLC.
Digestions were performed in (a) 10 mM HCl, pH 2.0, (b) 10 mM HCl, pH 2.0 + 154 mM NaCl, (c) 50 mM glycine, pH 3.2, (d) 50 mM glycine, pH 3.2 + 154 mM NaCl using an enzyme : substrate ratio of 1 : 10 at 37° C. The 15 min time-point was analysed by HPLC on a Brownlee Aquapore C4 column using a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at 215 nm. Intact long-R^3-IGF-I elutes at approximately 30 min under these conditions. The digestion fragment, FVN-R^3-IGF-I, is indicated by the arrow.

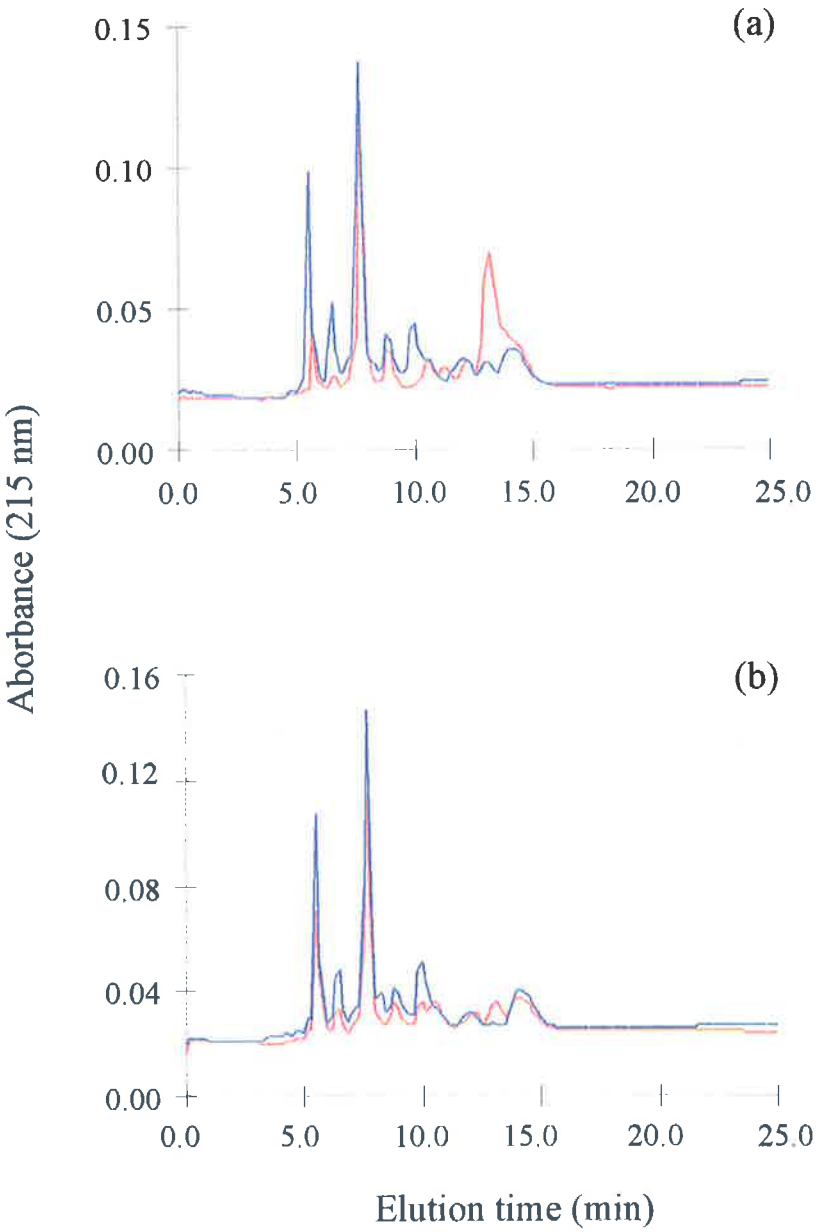


FIGURE 4.14
Reverse-phase HPLC elution profiles of long-R^3-IGF-I after 60 min pepsin digestion in HCl and glycine buffers.
Digestion of long-R^3-IGF-I in (a)10 mM HCl, pH 2.0, and (b) 50 mM glycine, pH 3.2, in the presence (blue) and absence (red) of 154 mM NaCl. Digestions were performed using an enzyme : substrate ratio of 1 : 10 for 60 min at 37° C followed by analysis on a Brownlee Aquapore C4 column using a gradient of acetonitrile in 0.1 % (v/v) TFA at 30° C. The gradient used was 0 to 20 % acetonitrile for 5 min followed by 20 to 40 % acetonitrile for 40 min, at a flow rate of 0.5 ml/min. The elution of protein was monitored at 215 nm. The digestion fragment, FVN-R^3-IGF-I, elutes at approximately 21 min under these conditions.

concentrations. This effect was also observed with long-R^3-IGF-I (Figure 4.13). Figure 4.14 is an example of pepsin digestion of long-R^3-IGF-I at the more complete degradation time-point of 60 min, enzyme to substrate ratio 1 : 10 (w/w). Comparison of the HPLC profiles between the digestion buffers did not suggest the presence of substantial "new" peptide fragments, indicative of alternative pepsin cleavage sites occurring due to buffer differences. Rather, the presence of peptide peaks in varying amounts, and their absence in some cases, is more likely a reflection of the different degradation rates occurring in these buffers. However, this would need to be further confirmed by analysis of the peptide fragments by mass spectrometry and amino acid sequencing.

4.4.6 Effect of enzyme : substrate ratio on peptide digestion

Pepsin concentration in the stomach lumen is known to vary considerably, with low concentrations in the unstimulated state and much higher amounts secreted following gastric stimulation (Jones *et al.*, 1993). Since rates of protein degradation are dependent on the ratio of enzyme : substrate, a comparison of the degradation rates of long-R^3-IGF-I and long-R^3A^{16}-IGF-I were made over a range of pepsin : peptide ratios, 20 : 1, 4 : 1 and 1 : 2.5. A competitive IGF-receptor binding assay was used to assess stability. At all enzyme : substrate ratios tested, long-R^3A^{16}-IGF-I was more stable than long-R^3-IGF-I but the differential in stability varied with enzyme concentration (Figure 4.15). Peptide digestion at the lowest enzyme : substrate ratio (1 : 2.5, w/w) was negligible for both peptides as shown by ≥100 % apparent recovery of receptor-binding activity over a 120 min incubation period. In the case of the analogue, long-R^3A^{16}-IGF-I, recovery of biological activity was actually greater than 100 %, presumably due to the rapid cleavage of the *N*-terminal extension peptide.

At the enzyme : substrate ratio of 4 : 1 (w/w), long-R^3-IGF-I was degraded rapidly with a half-life of 8 min. Long-R^3A^{16}-IGF-I was approximately 8-fold more stable resulting in a half-life of 63 min. In addition the mutated analogue showed an initial increase in receptor binding, which was again presumed to reflect cleavage of the extension peptide. At the highest enzyme : substrate ratio (20 : 1, w/w) the stability advantage of the long-R^3A^{16}-IGF-I

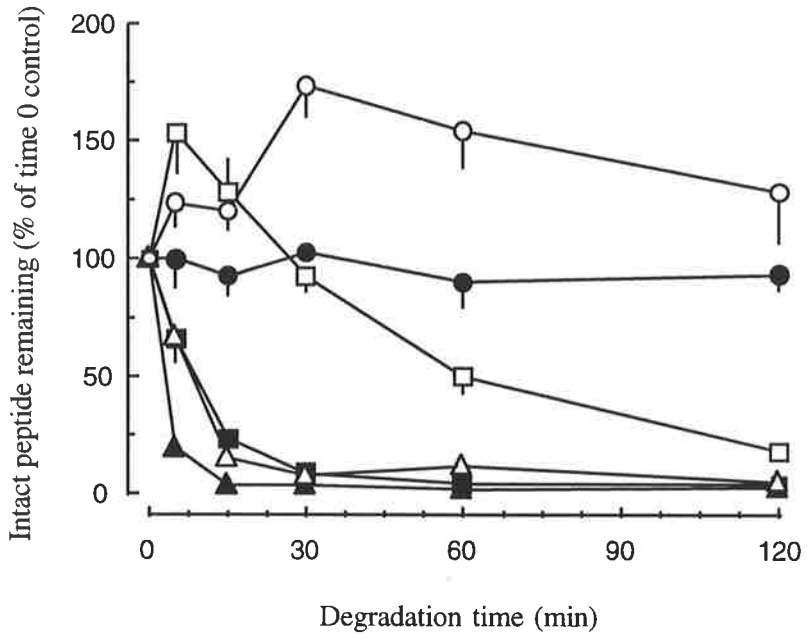


FIGURE 4.15

Effect of different pepsin : peptide ratios on the stability of long-R^3-IGF-I and long-R^3A^{16}-IGF-I in 10 mM HCl, pH 2.0.

Long-R^3-IGF-I (closed symbols) and long-R^3A^{16}-IGF-I (open symbols) were incubated with different amounts of purified porcine pepsin in 10 mM HCl, pH 2.0, at 37° C for the indicated times. The amount of intact peptide remaining was estimated in a competitive IGF-receptor binding assay, and expressed as a % of the time zero control. Values are means of triplicate determinations at each time point with SEM values of greater than 5 % indicated by bars. Enzyme : substrate ratios are represented as triangles for 20 : 1, squares for 4 : 1 and circles for 1 : 2.5.

analogue was reduced to only 2.7-fold, so that half-lives of the analogue and parent molecule were 8 and 3 min respectively.

4.5 DISCUSSION

4.5.1 Stability of IGF analogues in luminal flushings.

The rates of degradation of the IGF analogues were determined in stomach flushings from rat, pig and human sources. Gut flushings rather than *in vivo* ligated segments were used to assess the stability as this approach is presumed to reflect a close approximation to *in vivo* conditions. This method with both human and rat fluids, has previously been used by several groups (reviewed by Britton & Koldovsky, 1989). More recently, Xian *et al.* (1995) have shown that long-R^3-IGF-I is degraded with essentially identical half-lives in luminal flushings and *in vivo* ligated segments of the rat gastrointestinal tract.

Stomach flushings from the different species (rat, human and pig) varied in their ability to degrade the IGF analogues when standardised to contain the same activity against denatured haemoglobin. It is unlikely that these differences reflected variable contamination from duodenal enzymes since the incubations were carried out in the presence of aprotinin and PMSF, conditions that were shown to inhibit duodenal degradation of the peptides. Rather, the differences in degradation rate may reflect distinct substrate specificities of the major pepsin isoforms of the three species. Alternatively, human, pig and rat stomach may secrete a different mix of pepsin isozymes. Mammalian gastric secretions are known to contain several isoforms of pepsin in addition to the predominant A and C forms (Foltmann, 1981). For example, human pepsin has been reported to contain 20 distinct electrophoretic forms (Jones *et al.*, 1993), while pig stomach flushings contain a poorly characterised group of minor isozymes referred to as pepsin B (Ryle, 1970) and rat stomach contains a unique form, pepsin 1 (Furihata *et al*, 1980). The various isozymes show different pH optima as well as substrate specificity (Tang, 1970). Accordingly, the susceptibility of IGF-I analogues would be expected to vary depending on the precise mix of isozymes present in the stomach flushings of each species. Evidence for a mixture of gastric isozymes active against IGF-I was obtained by comparison of rates of IGF degradation by purified porcine pepsin compared to

pig stomach flushings. When standardised to the same activity against haemoglobin, purified pepsin (EC 3.4.23.1; pepsin A) degraded long-R^3-IGF-I or long-R^3A^{16}-IGF-I more rapidly than flushings from the pig stomach.

The mutated IGF-I analogues were specifically designed for increased stability against purified pepsin. However, these studies have demonstrated that the long-R^3-IGF-I analogues are also stabilised in stomach flushings when examined under the standardised conditions of glycine buffer, pH 3.2 containing 154 mM NaCl. In rat stomach flushings the relative stabilities of the complete range of long-R^3-IGF-I analogues were comparable to the results obtained with purified porcine pepsin in Chapter 3. The variant with the Phe16Ala mutation (long-R^3A^{16}-IGF-I) showed the greatest pepsin stability while the other analogues with the Leu10Val, Met59Gln, Phe25Leu or Asp53Glu mutations had less pronounced effects. As in the previous chapter, long-R^3L^{25}-IGF-I showed a slight increase, and long-R^3E^{53}-IGF-I a small decrease in stability relative to the parent peptide. Comparisons of stomach flushings across the three species demonstrated an approximate 2-fold higher stability of long-R^3A^{16}-IGF-I compared to the parent long-R^3-IGF-I peptide. Hence, the protein engineering approach used was successful in achieving a generic enhancement of IGF stability against gastric digestion without substantial loss of IGF bio-activity. The incorporation of more than one of these mutations into long-R^3-IGF-I would most likely further improve gastric stability.

Xian *et al.* (1995) have shown that both IGF-I and long-R^3-IGF-I are rapidly degraded in the duodenum to TCA soluble small peptides. Comparison of the degradation rates of long-R^3-IGF-I and the analogues in rat duodenal flushings, confirmed that long-R^3-IGF-I had a limited stability in the duodenum. Furthermore, it was shown that the additional mutations that were incorporated into long-R^3-IGF-I had only a minor stabilising effect in this area of the gastrointestinal tract.

4.5.2 Effect of enzyme concentrations, buffer and salt on peptide digestion

Notwithstanding the demonstrated stability advantage of the long-R^3A^{16}-IGF-I analogue over the parent long-R^3-IGF-I, the stability of both peptides, and particularly the mutated analogue, varied with changes in enzyme : substrate ratio, pH and salt.

Long-R^3A^{16}-IGF-I showed an 8-fold greater half-life than the parent molecule when incubated with purified porcine pepsin at an enzyme : peptide ratio of 4 : 1 (w/w). However, the differential in stability was diminished at higher enzyme concentrations, so that long-R^3A^{16}-IGF-I was only 2-fold more stable than long-R^3-IGF-I at an enzyme : peptide ratio of 20 : 1 (w/w). This suggests that the stability advantage of long-R^3A^{16}-IGF-I in the stomach *in vivo* would diminish under conditions of active pepsin secretion. This is supported by the findings with pig stomach flushings, where long-R^3A^{16}-IGF-I was two-fold more stable than the parent peptide in diluted flushings, but showed much less stability advantage in undiluted flushings, with half-lives of 9 and 7 min, respectively.

The gastric juice of a fasted human has Na^+ concentrations of 19 to 70 mM with other ions such as potassium, ammonium, magnesium and calcium also present although in minor concentrations (Leach, 1961). The addition of 154 mM NaCl to the digestion buffer markedly increased degradation of the long-R^3-IGF-I peptides. Mechanisms of this decreased stability are not clear, although the salt may interfere with the charge interactions in the long-R^3-IGF-I molecule. The proposed structure for IGF-I predicts salt bridges on the surface of the molecule due to the network of charged groups (Blundell *et al.*, 1978, Sato *et al.*, 1993). Hence, interference with these bridges could cause a conformational change leading to increased susceptibility of the peptide to pepsin proteolysis at both 'major' and 'minor sites' of cleavage, as well as providing access to previously internal cleavage sites. However, preliminary peptide mapping experiments in the presence and absence of salt revealed similar patterns of digestion suggesting that the 'major' cleavage sites may become more accessible for pepsin proteolysis.

Since pepsin activity is pH-dependent (Schamowitz & Peterson, 1959; Furihata *et al.*, 1980) changes in the acidity of the stomach environment could also affect the stability of a peptide to degradation. Long-R^3-IGF-I and long-R^3A^{16}-IGF-I were degraded more rapidly by

porcine pepsin in glycine buffer (pH 3.2) than in HCl (pH 2.0). This result was surprising in that the purified pepsin A used in the study is generally considered to have a pH optimum of 2.0 (Foltmann, 1981). However, it is also known that the pH optima of pepsin isozymes can vary with different substrates (Schamowitz & Peterson, 1959; Pearson *et al.*, 1986). If so, then the results suggest that porcine pepsin A is more active against IGF analogues at a higher pH. Alternatively, the slower digestion at the lower pH may reflect autodigestion of pepsin. Pepsin is reported to undergo autolytic degradation more readily at lower pH, thereby reducing the effective enzyme concentration as well as causing the accumulation of low molecular weight products that further inhibit pepsin (Fruton, 1971). Additional explanations for this result may relate to differences in ionic strength between the buffers as eluded to above or pH-dependent conformational changes in the IGF analogues that may lead to an increased susceptibility to proteolysis at the higher pH. It is important to note that all NMR studies of IGF-I have been performed at low pH (Cooke *et al.*, 1991; Sato *et al.*, 1992 & 1993) and circular dichroism studies indicate that acid conditions alone are unlikely to alter the secondary structure of IGF-I (Hejnaes *et al.*, 1992).

4.5.3 Comparison of iodinated and unlabelled peptide stability

The stability of the IGF analogues was determined in rat stomach flushings using both unlabelled and iodinated peptides. Since the chloramine-T method results in iodination of multiple tyrosine residues in IGF-I, at positions 24, 31 and 60 (Schäffer *et al.*, 1993), the potential exists for interference in pepsin digestion. In particular, iodination of tyrosine 24 and 60 could be expected to interfere as these residues are adjacent to major pepsin cleavage sites in long-R^3-IGF-I, as shown in Chapter 2 and the study by Forsberg *et al.* (1990). However, the observations of similar stability of unlabelled and iodinated IGF-I analogues in rat stomach flushings implies that any structural modifications resulting from iodination have little effect on susceptibility to pepsin.

4.5.4 Enhancement in receptor binding activity

In 10 mM HCl, pH 2.0, long-R^3A^{16}-IGF-I showed an initial increase in receptor binding activity following exposure to pepsin. This was presumably due to the generation of a more biologically active peptide, FVN-R^3-IGF-I, which occurs as the first step in pepsin digestion (Chapter 2). However, long-R^3-IGF-I has an identical *N*-terminal extension peptide which is also rapidly cleaved during the first stages of degradation and yet showed no such enhancement in biological activity. Several possibilities may account for this observed difference. It is possible that FVN-R^3A^{16}-IGF-I may have an increased affinity for the placental membrane receptors compared to the corresponding long-R^3-IGF-I peptide, FVN-R^3-IGF-I. Alternatively, FVN-R^3-IGF-I may be degraded much more rapidly than its mutated counterpart, thereby masking any transient increase in receptor affinity. Indeed, in some experiments such as those described in Table 4.1 where longer half-lives were measured, a slight increase in activity was observed during the initial digestion of long-R^3-IGF-I.

4.5.5 Summary

In the previous chapter, the pepsin stabilities of five analogues of long-R^3-IGF-I containing single amino acid mutations were investigated *in vitro* using reverse-phase HPLC to monitor degradation. In this chapter, the initial findings have been extended to examine stability of the analogues under conditions that more closely reflect the stomach lumen *in vivo*. These studies have shown that although the stability advantage of long-R^3A^{16}-IGF-I is dependent of pH, salt concentration or enzyme : substrate ratio, it is consistently more stable than the parent long-R^3-IGF-I molecule against purified porcine pepsin or stomach flushings from several species.

CHAPTER FIVE

CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 GENERAL SUMMARY

At the commencement of the research for this PhD thesis, IGF-I and its analogue, long-R^3-IGF-I, had been shown to be potent growth factors for the gastrointestinal tract *in vivo*. Subcutaneous administration of IGF-I into rats selectively increased gut weight in a dose-dependent manner, with anabolic responses apparent throughout the entire gastrointestinal tract (Read *et al.*, 1992b). A limited number of studies had investigated oral or direct infusion of the peptides into the lumen, and these showed some evidence that low doses of IGF-I increase intestinal brush border enzyme activity and mucosal growth (Young *et al.*, 1990; Olanrewaju *et al.*, 1992). Although these findings may have significance for the development of new treatments for gut disease, IGF-I has been shown to be rapdily degraded in all areas of the gastrointestinal tract (Koldovsky *et al.*, 1992; Xian *et al.*, 1995) thus limiting its potential effectiveness. Consequently, a gut-stable form of IGF-I could have potential in therapeutic applications. Thus, the goal of my research was to produce pepsin-resistant mutants of long-R^3-IGF-I which retained biological activity.

Five putatively pepsin-resistant analogues of long-R^3-IGF-I were constructed using site directed mutagenesis. These analogues contained single-point mutations in the IGF-I moiety; Leu10Val, Phe16Ala, Phe25Leu, Asp53Glu and Met59Gln. Biological assays of the analogues showed the mutations Leu10Val and Phe25Leu had reduced type 1 receptor binding, whereas Phe16Ala, Asp53Glu and Met59Gln had a limited effect. Relative binding of the analogues to IGFBPs, as secreted by L6 myoblasts, showed the Phe25Leu and Met59Gln mutants had affinities similar to that of the parent molecule, the Asp53Glu analogue had a slightly increased affinity, whereas both Leu10Val and Phe16Ala showed marked decreases in IGFBP affinity. Bio-activity, as assessed by stimulation of protein synthesis, showed the Met59Gln and Asp53Glu mutants were slightly more potent than long-R^3-IGF-I, whereas Phe16Ala had a slight decrease in potency, and the mutants Leu10Val and Phe25Leu had significantly decreased bio-activity. Degradation studies of the mutants showed the analogue

containing the Phe16Ala mutation had the greatest pepsin stability, being stabilised against purified porcine pepsin approximately 10-fold compared to long-R^3-IGF-I. The other mutants had either stabilities that were intermediate between the Phe16Ala mutant and long-R^3-IGF-I or approximately equivalent to the parent molecule.

This research has shown that site-directed mutagenesis could effectively be used to generate pepsin-stable IGF-I variants with retention of bio-activity.

5.2 THERAPEUTIC APPLICATIONS

In this study, mutants of IGF-I were produced with pepsin-resistance enhanced by up to 10-fold when digested *in vitro* with purified porcine pepsin. The stability advantage of these analogues, however, diminished under conditions that more closely reflected the stomach environment. For example, the long-R^3A^{16}-IGF-I analogue, had markedly increased pepsin-stability *in vitro* to purified porcine pepsin, but this was reduced to approximately 2-fold, compared to the parent material, when degradation studies were performed using luminal gut flushings. This relatively small increase in pepsin-stability under conditions that approximate the stomach environment, will consequently limit the therapeutic potential of this analogue. Although the selected amino acid substitutions did enhance pepsin-stability in some cases, presumably due to limiting pepsin cleavage at the substituted peptide bond, these mutations did not prevent further degradation of the analogues by the enzyme. Rather, the mutations probably increased the pepsin-susceptibility of alternative 'major' and 'minor' cleavage sites. Consequently, as the long-R^3-IGF-I analogues produced in this study contained only single-point mutations, the incorporation of several of the selected amino acid substitutions should most likely further enhance the gastric stability of IGF-I by limiting cleavage at a number of 'major' proteolytic sites. However, such variants also have potential for multiple loss of bio-activity. Therefore, considerably more research is required in this area to determine if IGF-stability can be further enhanced in the stomach environment whilst retaining biological activity and thus enabling its use as a gastrointestinal therapeutic.

Further research, such as extending the proteolytic resistance of IGF-I to other gastrointestinal enzymes, may be beneficial in the treatment of a variety of gut diseases. For instance, if peptide stability could be extended to the intestine this may lead to the treatment of diseases involving intestinal atrophy, as has been shown in short bowel syndrome patients where parenteral nutrition is required to maintain total nitrogen balance (Johnson *et al.*, 1975; Levine, 1991). Since administration of IGF-I and its analogues has been shown to significantly enhance mucosal growth (Lemmey *et al.*, 1991 & 1994; Vanderhoof *et al.*, 1992; Read *et al.*, 1992; Steeb *et al.*, 1994) IGF-I may have potential applications in these conditions. The research in this thesis has shown that proteolytic stability of IGF-I can be enhanced with selected mutations and this consequently is applicable to the intestinal regions of the gut. Intestinal therapeutics of IGF-I may not necessarily have to contain the pepsin-stabilising mutations, since the stability required for passage through the stomach can be achieved by other methods, such as the use of polymer-capsules (Langer & Moses, 1991; Florence & Jani, 1994). In fact, it is probably more achievable to enhance peptide-stability to selected gut regions since this will minimise the number of amino acid substitutions required, thereby decreasing the potential to alter tertiary structure and hence bio-activity. Furthermore, due to the complexity of enzymes in the gastrointestinal tract, the production of a total gut-stable IGF-I analogue is unlikely.

5.3 ADDITIONAL RESEARCH

In addition to producing and characterising long-R^3-IGF-I variants containing multiple point mutations, several other lines of research from this study would also be interesting to follow.

Site directed mutagenesis has been used in this, and numerous other projects (Lu *et al.*, 1991; Regnstrom *et al.*, 1994) to investigate the structural and functional significance of particular residues within a peptide or protein. If an amino acid mutation results in a structural change in the conformation of the peptide, due to a disturbance in the protein fold or tertiary characteristics (locally as well as globally), this can affect bio-activity. Consequently, amino acid substitutions affecting bio-activity could be the result of a

conformational change occurring in the peptide rather than an affect due to the alteration of the functional group on the amino acid. Although the mutations in this study were chosen to limit such conformational changes in the long-R^3-IGF-I molecule, it can not be excluded that structural changes did not occur in the analogues and hence these are the cause of the observed alterations in biological activity. Additional research investigating the structural integrity of all the variants, such as using two-dimensional NMR and circular dichroism analysis, would be interesting especially in the case of the Leu10Val mutation which was not as effective as predicted. Examples where altered structural integrity affects biological properties, has been shown recently by Jansson & Nilsson, (1995). These workers constructed analogues of IGF-I with single amino acid substitutions in the *B*-domain of the molecule. The variants, containing either a Val11Ala, Asp12Ala, Gln15Ala, Gln15Glu or Phe16Ala mutation, had reduced affinity to a soluble form of the type 1 IGF-receptor and altered binding to IGFBP-1. In addition, circular dichroism analysis of the peptides suggested that in particular the Val11Ala and Phe16Ala mutants had a lowered α-helical content compared to native IGF-I. Hence, these results indicate that the changed binding properties of the IGF-I variants are not only due to altered interactions from amino acid substitutions but also from structural changes, and furthermore, even single amino acid mutations can alter the tertiary structure of IGF-I. This reported research (Jansson & Nilsson, 1995) suggests that structural changes, both secondary and tertiary, have probably also occurred in some of the variants I produced, in particular the long-R^3-A^{16}-IGF-I and long-R^3-V^{10}-IGF-I analogues. Perhaps these analogues can also undergo conformational changes depending on buffer conditions, and this may explain the difference in pepsin-stability observed with long-R^3A^{16}-IGF-I in the various degradation buffers.

The construction of the analogues as single-site mutations enabled a structural and functional study on the long-R^3-IGF-I molecule, with implications for the formation of similar analogues of IGF-I. The complete NMR structure of long-R^3-IGF-I has not yet been deduced, although preliminary studies indicate the *N*-terminal extension peptide has random and limited structure (Dr J. Carver, University of Wollongong, NSW, Australia, personal communication). However, for a direct structure and function comparison of the substitutions

produced in this study, the biological activities would have to be assessed in the absence of the 13 amino acid fusion-peptide. In addition, this would also allow a comparative study of the effects of substitutions between IGF-I and long-R^3-IGF-I. Further research in this area may elucidate whether the FVN-R^3A^{16}-IGF-I analogue has increased IGF-receptor affinity compared to long-R^3A^{16}-IGF-I. This information may explain the enhancement in biological activity shown with long-R^3A^{16}-IGF-I compared to long-R^3-IGF-I during pepsin digestion.

5.4 FUTURE DIRECTIONS

Although the site-directed mutagenesis approach used in this study was effective in producing some IGF-I variants with enhanced pepsin-resistance, this was a time-consuming method for generating potentially stable analogues. If the efficiency of the selection and generation procedure for analogues could be increased then this would be an interesting line of research to pursue. A method such as random mutagenesis may be applicable. For example, if random mutagenesis could be directed at each of the amino acids selected for substitution then a library of variants would be generated. Subsequent screening of these variants for either proteolytic resistance and/or retained IGF-receptor binding could evaluate the potential of each substitution and hence whether the substitution was appropriate for applying to a putative gut-stable IGF-I analogue. Use of a random mutagenesis method such as this may help identify more suitable amino acids to substitute at Phe25 which maintain type 1 IGF- receptor affinity. Although, the Phe25Leu mutation I selected during this study did confer a degree of pepsin resistance, the IGF-receptor binding was reduced. Similarly, with respect to the Leu10-Val11 bond, it would be interesting to determine amino acid substitutions which conferred pepsin stability without adverse effects on biological activity. Furthermore, although the Phe16Ala mutation selected during this research effectively enhanced pepsin stability and retained bio-activity, are other amino acids more effective than the selected Ala residue? Recently, similar approaches have been used by other workers. For example, Graham *et al.* (1993) constructed a library of random substitutions at residues likely to influence the primary specificity of alpha-lytic protease. The active enzymes in the library were then screened with a range of synthetic substrates in order to evaluate their primary

cleavage preferences. Mutant enzymes with greatly increased proteolytic activity were also found. Similarly, Lowman & Wells (1993) selected high affinity variants of human GH by randomly mutating residues shown to be important for receptor binding. The mutated proteins, displayed on filamentous phagemid particles, were sorted *in vitro* for binding to immobilised receptors. After three to seven rounds of binding enrichments, variants were isolated that contained two to four mutations and exhibited 3- to 6-fold improvements in binding affinity. By combining affinity enhanced mutants, Lowman & Wells produced a GH variant with 15 substitutions that bound the extracellular domain of the GH receptor approximately 400-fold tighter than wild-type GH.

It is possible that gut effects in response to intraluminal delivery of IGF-I differ from those seen after systemic administration of IGF-I. To date, most studies have assessed the efficiency of IGF peptides on gastrointestinal growth and function when administered subcutaneously. In addition to the stimulation of gastrointestinal tissue, the growth of non-gut organs are also stimulated when the IGF peptides are administered via this route. To assess the effects of an orally administered IGF-I and to determine if these effects are limited to mucosal stimulation or include growth associated with other areas, requires the survival of the peptide in the gut lumen. Consequently, as IGF-I is rapidly degraded in the gastrointestinal tract (Xian *et al.*, 1995), a proteolytic-resistant IGF-I could benefit these studies by prolonging the action of IGF-I on the gastrointestinal mucosa.

As the gastrointestinal tract contains a number of digestive enzymes with varied proteolytic preferences and activity, perhaps a future direction for oral peptide therapeutics is in the area of peptidomimetics. These are compounds with altered chemical structures where the peptide-backbone is replaced by other organic moieties. These compounds, however, still maintain the ability to interact with a specific peptide receptor by retaining essential chemical functionalities and the ability to display them in their characteristic three-dimensional pattern (reviewed by Hruby, 1993; Marshall, 1993). Consequently, if a non-peptide mimetic of IGF-I could be designed, the retained bio-activity combined with the proteolytic stability, due to the removal of peptide-bonds, would result in a potent gastrointestinal therapeutic.

REFERENCES

Adamo, M., Lowe, W. L. Jr., LeRoith, D. & Roberts, C. T. Jr. (1989) Insulin-like growth factor I messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development of the rat. *Endocrinology* **124** : 2737-2744.

Adamo, M. L., Bach, M. A., Roberts, C. T. Jr. & LeRoith, D. (1991) Regulation of insulin, IGF-I and IGF-II gene expression. In: *Insulin-like Growth Factors: Molecular and Cellular Aspects* (LeRoith, D. ed.), pp. 271-303, CRC Press, Boca Raton.

Adashi, E. Y., Resnick, C. E., Svoboda, M. E. & Van Wyk, J. J. (1984) A novel role for somatomedin-C in the cytodifferentiation of the ovarian granulosa cell. *Endocrinology* **115** : 1227-1229.

Albiston, A. L., Taylor, R. G., Herington, A. C., Beveridge, D. J. & Fuller, P. J. (1992) Divergent ileal IGF-I and IGFBP-3 gene expression after small bowel resection: a novel mechanism to amplify IGF action? *Molecular and Cellular Endocrinology* **83** : R17-R20.

Allen, A., Pearson, J. P., Blackburn, A., Coan, R. M., Hutton, D. A. & Mall, A. S. (1988) Pepsins and the mucus barrier in peptic ulcer disease. *Scandinavian Journal of Gastroenterology* **23** (Suppl. 146) : 50-57.

Anson, M. L. (1938) The extinction of pepsin, trypsin, papain and cathepsin with hemoglobin. *The Journal of General Physiology* **22** : 79-89.

Arafah, B. M. (1991) Insulin-like growth factor-I receptors in the rat adrenals. *The Journal of Laboratory and Clinical Medicine* **118** : 531-537.

Arrieta-Molero, J. F., Aleck, K., Sinha, M. K., Brownscheidle, C. M., Shapiro, L. J. & Sperling, M. A. (1982) Orally administered liposome-entrapped insulin in diabetic animals. A critical assessment. *Hormone Research* **16** : 249-256.

Auffret, C. A. & Ryle, A. P. (1979) The catalytic activity of pig pepsin C towards small synthetic substrates. *Biochemical Journal* **179** : 239-246.

Axelsson, C. K. (1992) Clinical implications of serum pepsinogen and progastricsin in man. *Scandinavian Journal of Clinical and Laboratory Investigation* **52** (Suppl. 210) : 81-96.

Bach, M. A., Roberts, C. T. Jr., Smith, E. P. & LeRoith, D. (1990) Alternative splicing produces messenger RNAs encoding insulin-like growth factor-I prohormones that are differentially glycosylated *in vitro*. *Molecular Endocrinology* **4** : 899-904.

Bach, M. A., Hsieh, S., Sakano, K., Fujiwara, H., Perdue, J. F. & Rechler, M .M. (1993) Binding of mutants of human insulin-like growth factor II to insulin-like growth factor binding proteins 1-6. *The Journal of Biological Chemistry* **268** : 9246-9254.

Bagley, C. J., May, B. L., McNamara, P. J., Ross, M., Francis, G. L., Ballard, F. J. & Wallace, J. C. (1989) A key functional role for the insulin-like growth factor I N-terminal pentapeptide. *Biochemical Journal* **259** : 665-671.

Ballard, F. J., Read, L. C., Francis, G. L., Bagley, C. J. & Wallace, J. C. (1986) Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts. *Biochemical Journal* **233** : 233-230.

Ballard, F. J., Francis, G. L., Ross, M., Bagley, C. J., May, B. & Wallace, J. C. (1987) Natural and synthetic forms of insulin-like growth factor-I (IGF-I) and the potent derivative, destripeptide IGF-I: Biological activities and receptor binding. *Biochemical and Biophysical Research Communications* **149** : 398-404.

Ballard, F. J., Johnson, R. J., Owens, P. C., Francis, G. L., Upton, F. M., McMurtry, J. P. & Wallace, J. C. (1990) Chicken insulin-like growth factor-I: amino acid sequence, radioimmunoassay and plasma levels between strains and during growth. *General and Comparative Endocrinology* **79** : 459-468.

Ballard, F. J., Knowles, S. E., Walton, P. E., Edson, K., Owens, P. C., Mohler, M. A. & Ferraiolo, B. L. (1991a) Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I), IGF-II and des(1-3)IGF-I in rats. *Journal of Endocrinology* **128** : 197-204.

Ballard, F. J., Tomas, F. M., Read, L. C., Knowles, S. E., Owens, P. C., Lemmey, A. B., Martin, A. A., Wells, J. R. E., Wallace, J. C. & Francis, G. L. (1991b) Effects of IGF-I and IGF analogs on growth during catabolic states in rats. In: *Modern Concepts of Insulin-like Growth Factors* (Spencer, E. M. ed.), pp. 617-627, Elsevier Science Publishing Co. Inc., London.

Bass, S. H., Mulkerrin, M. G. & Wells, J. A. (1991) A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. *Proceedings of the National Academy of Sciences U.S.A.* **88** : 4498-4502.

Baxter, R. C. (1986) The somatomedins: insulin-like growth factors. *Advances in Clinical Chemistry* **25** : 49-115.

Baxter, R. C. & Martin, J. L. (1989) Binding proteins for the insulin-like growth factors: structure, regulation and function. *Progress in Growth Factor Research* **1** : 49-68.

Baxter, R. C., Martin, J. L. & Handelsman, D. J. (1984a) Identification of human semen insulin-like growth factor-I/somatomedin-C immunoreactivity and binding protein. *Acta Endocrinologica* **106** : 420-427.

Baxter, R. C., Zaltman, Z. & Turtle, J. R. (1984b) Immunoreactive somatomedin-C/insulin-like growth factor I and its binding protein in human milk. *Journal of Clinical Endocrinology and Metabolism* **59** : 955-959.

Baxter, R. C., Bayne, M. L. & Cascieri, M. A. (1992) Structural determinants for binary and ternary complex formation between insulin-like growth factor-I (IGF-I) and IGF binding protein-3. *The Journal of Biological Chemistry* **267** : 60-65.

Bayne, M. L., Applebaum, J., Chicchi, G. G., Hayes, N. S., Green, B. G. & Cascieri, M. A. (1988) Structural analogs of human insulin-like growth factor I with reduced affinity for serum binding proteins and the type 2 insulin-like growth factor receptor. *The Journal of Biological Chemistry* **263** : 6233-6239.

Bayne, M. L., Applebaum, J., Underwood, D., Chicchi, G. G., Green, B. G., Hayes, N. S. & Cascieri, M. A. (1989) The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor. *The Journal of Biological Chemistry* **264** : 11004-11008.

Bayne, M. L., Applebaum, J., Chicchi, G. G., Miller, R. E. & Cascieri, M. A. (1990a) The roles of tyrosines 24, 31 and 60 in the high affinity binding of insulin-like growth factor-I to the type 1 insulin-like growth factor receptor. *The Journal of Biological Chemistry* **265** : 15648-15652.

Bayne, S. J., Thomsen, J., Skriver, L., Van den Brande, J. L., Hoogerbrugge, C. M. & Van Buul-Offers, S. C. (1990b) Confirming the primary structure of insulin-like growth factors 1 and 2 isolated from porcine plasma using mass analysis. *Peptide Research* **3** : 271-273.

Behringer, R. R., Lewin, T. M., Quaife, C. J., Palmiter, R. D., Brinster, R. L. & D'Ercole, A. J. (1990) Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice *Endocrinology* **127** : 1033-1040.

Bell, G. I., Stempien, M. M., Fong, N. M. & Rall, L. B. (1986) Sequences of liver cDNAs encoding two different mouse insulin-like growth factor-I precursors. *Nucleic Acids Research* **14** : 7873-7882.

Bell, G. I., Stempien, M. M., Fong, N. M. & Seino, S. (1990) Sequence of a cDNA encoding guinea pig IGF-I. *Nucleic Acids Research* **18** : 4275.

Beukers, M. W., Oh, Y., Zhang, H., Ling, N. & Rosenfeld, R. G. (1991) [Leu^{27}] insulin-like growth factor II is highly selective for the type-II IGF receptor in binding, crosslinking and thymidine incorporation experiments. *Endocrinology* **128** : 1201-1203.

Bhaumick, B. & Bala, M. (1987) Binding and degradation of insulin-like growth factors I and II by rat kidney membrane. *Endocrinology* **120** : 1439-1448.

Blum, W. F., Behringer, J., Weber, P. & Ranke, M. B. (1991a) Serum levels of insulin-like growth factors (IGF) I and II and IGF binding protein 3 depend on liver function. *Hormone Research* **35** (Suppl. 2) : 38.

Blum, W. F., Ranke, M. B., Kietzmann, K., Tönshoff, B. & Mehls, O. (1991b) Growth hormone resistance and inhibition of somatomedin activity by excess insulin-like growth factor binding protein in uraemia. *Pediatric Nephrology* **5** : 539-544.

Blundell, T. L. & Humbel, R. E. (1980) Hormone families: pancreatic hormones and homologous growth factors. *Nature* **287** : 781-787.

Blundell, T. L., Bedarkar, S., Rinderknecht, E. & Humbel, R. E. (1978) Insulin-like growth factor: A model for tertiary structure accounting for immunoreactivity and receptor binding. *Proceedings of the National Academy of Sciences U.S.A.* **75** : 180-184.

Bordo, D. & Argos, P. (1991) Suggestions for "safe" residue substitutions in site-directed mutagenesis. *Journal of Molecular Biology* **217** : 721-729.

Boulware, S. D., Tamborlane, W. V., Matthews, L. S. & Sherwin, R. S. (1992) Diverse effects of insulin-like growth factor I on glucose, lipid and amino acid metabolism. *American Journal of Physiology* **262** : E130-E133.

Brinkman, U., Pai, L. H., Fitzgerald, D. J. & Pastan, I. (1992) Alteration of a protease-sensitive region of Pseudomonas exotoxin prolongs its survival in the circulation of mice. *Proceedings of the National Academy of Sciences U.S.A.* **89** : 3065-3069.

Britton, J. R. & Koldovsky, O. (1989) Development of luminal protein digestion: Implications for biologically active dietary polypeptides. *Journal of Pediatric Gastroenterology and Nutrition* **9** : 144-162.

Brown, A. L., Graham, D. E., Nissley, S. P., Hill, D. J., Strain, A. J. & Rechler, M. M. (1986a) Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. *The Journal of Biological Chemistry* **261** : 13144-13150.

Brown, L., Munoz, C., Siemer, L., Edelman, E. & Langer, R. (1986) Controlled release of insulin from polymer matrices. Control of diabetes in rats. *Diabetes* **35** : 692-697.

Buck, M. A., Olah, T. A., Weitzmann, C. J. & Cooperman, B. S. (1989) Protein estimation by the product of integrated peak area and flow rate. *Analytical Biochemistry* **182** : 295-299.

Caccia, P., Nitti, G., Cletini, O., Pucci, P., Ruoppolo, M., Bertolero, F., Valsasina, B., Roletto, F., Cristiani, C., Cauet, G., Sarmientos, P., Malorni, A & Marino, G. (1992) Stabilization of recombinant human basic fibroblast growth factor by chemical modification of cysteine residues. *European Journal of Biochemistry* **204** : 649-655.

Cao, Q-P., Duguay, S. J., Plisetskaya, E., Steiner, D. F. & Chan, S. J. (1989) Nucleotide sequence and growth hormone-regulated expression of salmon insulin-like growth factor I mRNA. *Molecular Endocrinology* **3** : 2005-2010.

Carlsson-Skwirut, C., Jörnvall, H., Holmgren, A., Andersson, C., Bergman, T., Lundquist, G., Sjögren, B. & Sara, V. R. (1986) Isolation and characterization of variant IGF-I as well as IGF-2 from adult human brain. *FEBS Letters* **201** : 46-50.

Carter, P. & Wells, J. A. (1988) Dissecting the catalytic triad of a serine protease. *Nature* **332** : 564-568.

Cascieri, M. A. & Bayne, M. L. (1994) Analysis of the interaction of IGF-I analogs with the IGF-I receptor and IGF binding proteins. In: *Current Directions in Insulin-like Growth Factor Research* (LeRoith, D. & Raizada, M. K. eds.), pp. 33-40, Plenum Press, New York.

Cascieri, M. A., Saperstein, R., Hayes, N. S., Green, B. G., Chicchi, G. G., Applebaum, J. & Bayne, M. L. (1988) Serum half-life and biological activity of mutants of human insulin-like growth factor I which do not bind to serum binding proteins. *Endocrinology* **123** : 373-381.

Cascieri, M. A., Chicchi, G. G., Applebaum, J., Green, B. G., Hayes, N. S. & Bayne, M. L. (1989) Structural analogs of human insulin-like growth factor (IGF) I with altered affinity for type 2 IGF receptors. *The Journal of Biological Chemistry* **264** : 2199-2202.

Cascieri, M. A., Chicchi, G. G. & Bayne, M. L. (1991) Characterization of the biological activity of IGF I analogs with reduced affinity for the IGF receptors and binding proteins. In: *Molecular Biology and Physiology of Insulin-like Growth Factors* (Raizada, M. K. & LeRoith, D. eds.), pp. 23-30, Plenum Press, New York.

Chaurasia, O. P., Marcuard, S. P. & Seidel, E. R. (1994) Insulin-like growth factor I in human gastrointestinal exocrine secretions. *Regulatory Peptides* **50** : 113-119.

Chen, B. & MacDonald, R. S. (1992) Responses of IGF binding proteins in rat serum following small bowel resection. *The FASEB Journal* **6** : A1079.

Chernausek, S. D., Beach, D. C., Banach, W. & Sperling, M. A. (1987) Characterisation of hepatic receptors for somatomedin C/insulin-like growth factor I and insulin in the developing human. *Journal of Clinical Endocrinology and Metabolism* **64** : 737-743.

Clemmons, D. R. & Underwood, L. E. (1994) Uses of human insulin-like growth factor-I in clinical conditions. *Journal of Clinical Endocrinology and Metabolism* **79** : 4-6.

Clemmons, D. R., Cascieri, M. A., Camacho-Hubner, C., McCusker, R. H. & Bayne, M. L. (1990) Discrete alterations of the insulin-like growth factor I molecule which alter its affinity for insulin-like growth factor binding proteins result in changes in bioactivity. *The Journal of Biological Chemistry* **265** : 12210-12216.

Clemmons, D. R., Demoff, M. L., Busby, W. H., Bayne, M. L. & Cascieri, M. A. (1992a) Competition for binding to insulin-like growth factor (IGF) binding protein-2, 3, 4 and 5 by the IGFs and IGF analogs. *Endocrinology* **131** : 890-895.

Clemmons, D. R., Smith-Banks, A. & Underwood, L. E. (1992b) Reversal of diet-induced catabolism by infusion of recombinant insulin-like growth factor-I (IGF-I) in humans. *Journal of Clinical Endocrinology and Metabolism* **75** : 234-238.

Cohick, W. S. and Clemmons, D. R. (1993) The insulin-like growth factors. *Annual Review of Physiology* **55** : 131-153.

Conover, C. A., Dollar, L. A., Hinz, R. L. & Rosenfeld, R. G. (1983) Insulin-like growth factor/somatomedin-C (IGF-I/Sm-C) and glucocorticoids synergistically regulate mitosis in competent human fibroblasts. *Journal of Cellular Physiology* **116** : 191-197.

Conover, C. A., Baker, B. K. & Hintz, R. L. (1989) Cultured human fibroblasts secrete insulin-like growth factor IA prohormone. *Journal of Clinical Endocrinology and Metabolism* **69** : 25-30.

Cook, J., J., Haynes, K. M. & Werther, G. A. (1988) Mitogenic effects of cultured human fibroblasts. Evidence for action via local insulin-like growth factor I production. *Journal of Clinical Investigation* **81** : 206-212.

Cooke, R. M., Harvey, T. S. & Campbell, I. D. (1991) Solution structure of human insulin-like growth factor I: A nuclear magnetic resonance and restrained molecular dynamics study. *Biochemistry* **30** : 5484-5491.

Costigan, D. C., Guyda, H. J. & Posner, B. I. (1988) Free insulin-like growth factor I (IGF-I) and IGF-II in human saliva. *Journal of Clinical Endocrinology and Metabolism* **66** : 1014-1018.

Cotterill, A. M. (1992) The therapeutic potential of recombinant human insulin-like growth factor-I. *Clinical Endocrinology* **37** : 11-16.

Cuatrecasas, P. (1972) Isolation of the insulin receptor of liver and fat-cell membranes. *Proceedings of the National Academy of Sciences U.S.A.* **69** : 318-322.

Culouscou, J. M., Remacle-Bonnet, M., Garrouste, F., Fantini, J., Marvaldi, J. & Pommier, G. (1990) Production of insulin-like growth factor II (IGF-II) and different forms of IGF-binding proteins by HT-29 human colon carcinoma cell line. *Journal of Cellular Physiology* **143** : 405-415.

Cunningham, B. C. & Wells, J. A. (1989) High resolution epitope mapping of hGH-receptor interaction by alanine-scanning mutagenesis. *Science* **244** : 1081-1085.

D'Ercole, A. J., Stiles, A. D. & Underwood, L. E. (1984) Tissue concentration of somatomedin C: Further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *Proceedings of the National Academy of Sciences U.S.A.* **81** : 935-939.

D'Ercole, A. J., Hill, D. J., Strain, A. J. & Underwood, A. J. (1986) Tissue and plasma somatomedin-C/insulin-like growth factor I concentrations in the human fetus during the first half of gestation. *Pediatric Research* **20** : 253-255.

Daughaday, W. H. & Rotwein, P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. *Endocrine Reviews* **10** : 68-91.

Daughaday, W. H., Hall, K., Raben, M. S., Salmon, W. D. Jr., Van den Brande, J. L. & Van Wyk, J. J. (1972) Somatomedin: Proposed designation for sulfation factor. *Nature* **235** : 107.

Daughaday, W. H., Hall, K., Salmon, W. D. Jr., Van den Brande, J. L. & Van Wyk, J. J. (1987) On the nomenclature of the somatomedins and insulin-like growth factors. *Journal of Clinical Endocrinology and Metabolism* **65** : 1075-1076.

Delafontaine, P., Lou, H., Harrison, D. G. & Bernstein, K. E. (1993) Sequence of a cDNA encoding dog insulin-like growth factor I. *Gene* **130** : 305-306.

Dixon, M. & Webb, E. C. (1964) Enzymes 2nd Ed., Longmans Green & Co Ltd, London.

Drakenberg, K., Sara, V. R., Falkmers, S., Gammeltoft, S., Maake, C. & Reinecke, M. (1993) Identification of IGF-I receptors in primitive vertebrates. *Regulatory Peptides* **43** : 73-81.

Dull, T. J., Gray, A., Hayflick, J. S. & Ullrich, A. (1984) Insulin-like growth factor II precursor gene organization in relation to insulin gene family *Nature* **310** : 777-781.

Ellerby, L. M., Escobar, W. A., Fink, A. L., Mitchinson, C. & Wells, J. A. (1990) The role of lysine-234 in beta-lactamase catalysis probed by site-directed mutagenesis. *Biochemistry* **29** : 5797-5806.

Emler, C. A. & Schalch, D. S. (1987) Nutritionally-induced changes in hepatic insulin-like growth factor I (IGF-I) gene expression in rats. *Endocrinology* **120** : 832-834.

Enberg, G., Carlqüist, M., Jörnvall, H. & Hall, K. (1984) The characterization of somatomedin A, isolated by microcomputer-controlled chromatography, reveals an apparent identity to insulin-like growth factor I. *European Journal of Biochemistry* **143** : 117-124.

Florence, A. T. & Jani, P. U. (1994) Novel oral drug formulations. Their potential in modulating adverse effects. *Drug Safety* **10** : 233-266.

Folkman, J., Szabo, S., Strovroff, M., McNeil, P., Li, W. & Shing, Y. (1991) Duodenal ulcer. Discovery of a new mechanism and development of angiogenic therapy that accelerates healing. *Annals of Surgery* **214** : 414-427.

Foltmann, B. (1981) Gastric proteinases - structure, function, evolution and mechanisms of action. *Essays in Biochemistry* **17** : 52-84.

Forbes, B. E., Szabo, L., Baxter, R. C., Ballard, F. J. & Wallace, J. C. (1988) Classification of the insulin-like growth factor binding proteins into three distinct binding categories according to their binding specificities. *Biochemical and Biophysical Research Communications* **157** : 196-202.

Forsberg, G., Palm, G., Ekebacke, A., Josephson, S. & Hartmanis, M. (1990) Separation and characterization of modified variants of recombinant human insulin-like growth factor I derived from a fusion protein secreted from *Escherichia coli*. *Biochemical Journal* **271** : 357-363.

Francis, G. L., Read, L. C., Ballard, F. J., Bagley, C. J., Upton, F. M., Gravestock, P. M. & Wallace, J. C. (1986) Purification and partial sequence analysis of insulin-like growth factor-I from bovine colostrum. *Biochemical Journal* **8** : 207-213.

Francis, G. L., Upton, F. M., Ballard, F. J., McNeil, K. A. & Wallace, J. C. (1988) Insulin-like growth factors I and II in bovine colostrum: sequence and biological activities compared to those of a potent truncated form. *Biochemical Journal* **251** : 95-103.

Francis, G. L., McNeil, K. A., Wallace, J. C., Ballard, F. J. & Owens, P. C. (1989a) Sheep insulin-like growth factors I and II: sequences, activities and assays. *Endocrinology* **124** : 1173-1183.

Francis, G. L., Owens, P. C., McNeil, K. A., Wallace, J. C. & Ballard, F. J. (1989b) Purification, amino acid sequences and assay cross-reactivities of porcine insulin-like growth factor-I and -II. *Journal of Endocrinology* **122** : 681-687.

Francis, G. L., Ross, M., Ballard, F. J., Milner, S. J., Senn, C., McNeil, K. A., Wallace, J. C., King, R. & Wells, J. R. E. (1992) Novel recombinant fusion protein analogues of insulin-like growth factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for enhanced biological activity. *Journal of Molecular Endocrinology* **8** : 213-223.

Francis, G. L., Aplin, S. A., Milner, S. J., McNeil, K. A., Ballard, F. J. & Wallace, J. C. (1993) Insulin-like growth factor (IGF)-II binding to IGF-binding proteins is modified by deletion of the N-terminal hexapeptide or substitution of arginine for glutamate-6 in IGF-II. *Biochemical Journal* **293** : 713-719.

Froesch, E. R., Bürgi, H., Ramseiler, E. B., Bally, P. & Labhart, A. (1963) Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiological significance. An insulin assay with adipose tissue of increased precision and specificity. *Journal of Clinical Investigation* **42** : 1816-1834.

Froesch, E. R., Guler, H-P., Schmid, C., Binz, K & Zapf, J. (1990) Therapeutic potential of insulin-like growth factor I. *Trends in Endocrinology and Metabolism* **1** : 254-260.

Froesch, E. R., Schmid, Ch., Schwander, J. & Zapf, J. (1985) Actions of insulin-like growth factors. *Annual Review of Physiology* **47** : 443-467.

Fruton, J. S. (1970) The specificity and mechanism of pepsin action. *Advances in Enzymology* **33** : 401-443.

Fruton, J. S. (1971) Pepsin. In: *The Enzymes 3rd Ed.* Hydrolysis: Peptide Bonds Vol. 3 (Boyer, P. D. ed.), pp. 120-164, Academic Press, New York.

Fruton, J. S. (1974) The active site of pepsin. *Accounts of Chemical Research* **7** : 241-246.

Furihata, C., Saito, D., Fujiki, H., Kanai, Y., Matsushima, T. & Sugimura, T. (1980) Purification and characterization of pepsinogens and a unique pepsin from rat stomach. *European Journal of Biochemistry* **105** : 43-50.

Goldstein, R. H., Poliks, C. F., Pilch, P. F., Smith, B. D. & Fine, A. (1989) Stimulation of collagen formation by insulin and insulin-like growth factor I in cultures of human lung fibroblasts. *Endocrinology* **124** : 964-970.

Gowan, G. K., Hampton, B., Hill, D. J., Schlueter, R. J. & Perdue, J. F. (1987) Purification and characterization of a unique high molecular weight form of insulin-like growth factor II. *Journal of Endocrinology* **121** : 449-458.

Graham, L. D., Haggett, K. D., Jennings, P. A., Le Brocque, D. S., Whittaker, R. G. & Schober, P. A. (1993) Random mutagenesis of the substrate binding site of a serine protease can generate enzymes with increased activities and altered primary specificities. *Biochemistry* **32** : 6250-6258.

Grant, M., Jerdan, J. & Merimee, T. J. (1987) Insulin-like growth factor-I modulates endothelial cell chemotaxis. *Journal of Clinical Endocrinology and Metabolism* **65** : 370-371.

Gray, G. M. & Cooper, H. L. (1971) Protein digestion and absorption. *Gastroenterology* **61** : 535-544.

Grimble, G. K. & Silk, D. B. A. (1989) Peptides in human nutrition. *Nutrition Research Reviews* **2** : 87-108.

Grossman M. I. (1980) Peptic ulcer: the pathophysiological background. *Scandinavian Journal of Gastroenterology* **15** (Suppl. 58) : 7-16.

Grosvenor, C. E., Picciano, M. F. & Baumrucker, C. R. (1992) Hormones and growth factors in milk. *Endocrine Reviews* **14** : 710-728.

Guler, H-P., Zapf, J. & Froesch, E. R. (1987) Short term metabolic effects of recombinant human insulin-like growth factor-I in healthy adults. *New England Journal of Medicine* **317** : 137-140.

Guler, H-P., Zapf, J., Scheiwiller, E. & Froesch, E. R. (1988) Recombinant human insulin-like growth factor I stimulates growth and has distinct effects on organ size in hypophysectomized rats. *Proceedings of the National Academy of Sciences U.S.A.* **85** : 4889-4893.

Guler, H-P., Schmid, C., Zapf, J. & Froesch, E R. (1990a) Effects of insulin-like growth factor-I in man. *Acta Paediatrica Scandinavica Supplement* **367** : 52-54.

Guler, H-P., Zapf, J., Schmid, C, Froesch, E. R. (1990b) Effects of rhIGF-I in man: possible therapeutic usefulness in states characterized by relative insulin resistance and in kidney disease. In: *Growth factors: From Genes to Clinical Application* (Sara, V. R. ed.), pp. 217-226, Raven Press, New York.

Han, V. K. M., D'Ercole, A. J. & Lund, P. K. (1987a) Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science* **236** : 193-197.

Han, V. K. M., Hill, D. J., Strain, A. J., Towle, A. C., Lauder, J. M., Underwood, L. E. & D'Ercole, A. J. (1987b) Identification of somatomedin/insulin-like growth factor immunoreactive cells in the human fetus *Pediatric Research* **22** : 245-249.

Han, V. K. M., Lund, P. K., Lee, D. C. & D'Ercole, A. J. (1988) Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization and tissue distribution. *Journal of Clinical Endocrinology and Metabolism* **66** : 422-429.

Hase, S., Nakazawa, S., Tsukamoto, Y. & Segawa, K. (1989) Effects of prednisolone and human epidermal growth factor on angiogenesis in granulation tissue of gastric ulcer induced by acetic acid. *Digestion* **42** : 135-142.

Haselbacher, G & Humbel, R. (1982) Evidence for two species of insulin-like growth factor II (IGF II and "big" IGF-II) in human spinal fluid. *Endocrinology* **110** : 1822-1824.

Hashimoto, A & Kawada, J.(1979) Effects of oral administration of positively charged insulin liposomes on alloxan diabetic rats: preliminary study. *Endocrinologica Japonica* **26** : 337-344.

Hatch, T., Branski, D., Krasner, J. & Lebenthal, E (1979) Effects of postnatal malnutrition on pancreatic zymogen enzymes in the rat. *The American Journal of Clinical Nutrition* **32** : 1224-1230.

Heinz-Erian, P. (1992) Gastrointestinal function in healthy children: significance of regulatory gut peptides. In: *Regulatory Gut Peptides in Paediatric Gastroenterology and Nutrition* Frontiers in Gastrointestinal Research Vol. 21 (Heinz-Erian, P., Deutsch, J., Granditsch, G. eds.), pp 103-114, Karger, Basel.

Hejnaes, K. R., Bayne, S., Norskov, L., Sorensen, H. H., Thomsen, J., Schaffer, L., Wollmer, A. & Skriver, L. (1992) Development of an optimized refolding process for recombinant Ala-Glu-IGF-I. *Protein Engineering* **5** : 797-806.

Henschel, M. J., Newport, M. J. & Parmar, V. (1987) Gastric proteases in the human infant. *Biology of the Neonate* **52** : 268-272.

Hernandez, E. R., Roberts, C. T. Jr., LeRoith, D. & Adashi, E. Y. (1989) Rat ovarian insulin-like growth factor I (IGF-I) gene expression is granulosa cell specific: 5' untranslated mRNA variant representation and hormonal regulation. *Endocrinology* **125** : 572-574.

Hernandez, E. R., Roberts, C. T. Jr., Hurwitz, A., LeRoith, D. & Adashi, E. Y. (1990) Rat ovarian insulin-like growth factor II gene expression is theca-interstitial cell-exclusive: hormonal regulation and receptor distribution. *Endocrinology* **127** : 3249-3251.

Herndon, D. N., Hayward, P. G., Rutan, R. L. & Barrrow, R. E. (1992) Growth hormones and factors in surgical patients. *Advances in Surgery* **25** : 65-97.

Hill, D. J., Crace, C. J., Strain, A. J. & Milner, R. D. G. (1986) Regulation of amino acid uptake and deoxyribonucleic acid synthesis in isolated human fetal fibroblasts and myoblasts: effect of human placental lactogen, somatomedian-C, multiplication-stimulating activity and insulin. *Journal of Clinical Endocrinology and Metabolism* **62** : 753-760.

Hizuka, N., Takano, K., Shizume, K., Asakawa, K., Miyakawa, M., Tanaka, I. & Horikawa, R (1986) Insulin-like growth factor I stimulates growth in normal growing rats. *European Journal of Pharmacology* **125** : 143-146.

Hizuka, N., Takano, Tanaka, I., K., Asakawa, K., Miyakawa, M., Horikawa, R & Shizume, K. (1987) Demonstration of insulin-like growth factor-I in human urine. *Journal of Clinical and Endocrinology and Metabolism* **64** : 1309-1312.

Hodgkinson, S. C., Napier, J. R., Davis, S. R., Patel, B. & Gluckman, P. D. (1989) Binding protein radioreceptor and biological activities of recombinant methionyl insulin-like growth factor-I variants. *Molecular and Cellular Endocrinology* **66** : 37-44.

Holly, J. M. P. & Wass, J. A. H. (1989) Insulin-like growth factors: autocrine, paracrine or endocrine? New perspectives of the somatomedin hypothesis in the light of recent developments. *Journal of Endocrinology* **122** : 611-618.

Hoyt, E. C., Van Wyk, J. J. & Lund, P. K. (1988) Tissue and development specific regulation of a complex family of rat insulin-like growth factor I messenger ribonucleic acids. *Molecular Endocrinology* **2** : 1077-1086.

Hruby, V. J. (1993) Conformational and topographical considerations in the design of biologically active peptides. *Biopolymers* **33** : 1073--1082.

Huang, K. F., Chung, D. H. & Herndon, D. N. (1993) Insulin like growth factor 1 (IGF-I) reduces gut atrophy and bacterial translocation after severe burn injury. *Archives of Surgery* **128** : 47-54.

Humbel, R. E. (1990) Insulin-like growth factors I and II. *European Journal of Biochemistry* **190** : 445-462.

Hunkapiller, M. W., Hewick, R. M. Dreyer, W. J. & Hood, L. E. (1983) High sensitivity sequencing with a gas phase sequenator. *Methods in Enzymology* **91** : 399-413.

Hynes, M. A., Van W. J., Brooks, P. J., D'Ercole, A. J., Jansen, M & Lund, P. K. (1987) Growth hormone dependence of somatomedin-C/insulin-like growth factor-I and insulin-like growth factor-II messenger ribonucleic acids. *Molecular Endocrinology* **1** : 233-242.

Izumi, T., White, M. F., Takashi, K., Takaku, F., Akanuma, Y. & Kusuga, M. (1987) Insulin-like growth factor I rapidly stimulates tyrosine phosphorylation of a Mr 185000 protein in intact cells. *The Journal of Biological Chemistry* **262** : 1282-1287.

Jacobs, S., Kull, F. C. Jr., Earp, H. S., Svobada, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1985) Somatomedin-C stimulates the phosphorylation of its own receptor. *The Journal of Biological Chemistry* **258** : 9581-9584.

James, R. & Bradshaw, R. A. (1984) Polypeptide growth factors. *Annual Review of Biochemistry* **53** : 259-292.

Jansen, E., Steenbergh, P. H., Van Schaik, F. M. A. & Sussenbach, J. (1992) The human IGF-I gene contains two cell type-specifically regulated promoters. *Biochemical and Biophysical Research Communications* **187** : 1219-1226.

Jansen, J., Van Buul-Offers, S. C., Hoogerbrugge, C. M. & Van den Brande, J. L. (1990) Effects of a single cleavage in insulin-like growth factors I and II on binding to receptors, carrier proteins and antibodies. *Biochemical Journal* **266** : 513-520.

Jansson, M. & Nilsson, B. (1995) Mutational analysis of insulin-like growth factor I protein-protein interactions. Proceedings of the Protein Society, First European Symposium, Davos, Switzerland, Abstract 342 (Forum B5).

Johnson, L. R., Copeland, E. M., Dudrick, S. J., Lichtenberger, L. M. & Castro, G. A. (1975) Structural and hormonal alterations in the gastrointestinal tract of parenterally fed rats. *Gastroenterology* **68** : 1177-1183.

Jones, A. T., Balan, K. K., Jenkins, S. A., Critchley, M. & Roberts, N. B. (1993) Assay of gastricsin and individual pepsins in human gastric juice. *Journal of Clinical Pathology* **46** : 254-258.

Jones, J. I. & Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* **16** : 3-34.

Kajimoto, Y. & Rotwein, P. (1990) Evolution of insulin-like growth factor-I (IGF-I): structure and expression of an IGF-I precursor from *Xenopus laevis*. *Molecular Endocrinology* **4** : 217-226.

Kajio, T., Kawahara, K. & Kato, K. (1992) Stabilization of basic fibroblast growth factor with dextran sulfate. *FEBS Letters* **306** : 243-246.

Karey, K. P., Marquardt, H. & Sirbasku, D. A. (1989) Human platelet-derived mitogens. 1. Identification of insulin-like growth factors I and II by purification and Na$^{\alpha}$amino acid sequence analysis. *Blood* **74** : 1084-1092.

Kasuga, M., Van Obberghen, E., Nissley, S. P. & Rechler, M. M. (1981) Demonstration of two subtypes of insulin-like growth factor receptors by affinity crosslinking. *The Journal of Biological Chemistry* **256** : 5350-5308.

Kay, J. & Dunn, B. M. (1992) Substrate specificity and inhibitors of aspartic proteinases. *Scandinavian Journal of Clinical and Laboratory Investigation* **52** (Suppl. 210) : 23-30.

Kemp, S. F., Kearns, G. L., Smith, W. G. & Elders, M. J. (1988) Effects of IGF-I on the synthesis and processing of glycosaminoglycan in cultured chick chondrocytes. *Acta Endocrinologica* **119** : 245-250.

Kidron, M., Barr-On, H., Berry, E. M. & Ziv, E. (1982) The absorption of insulin from various regions of the rat intestine. *Life Science* **31** : 2837-2841.

Kiess, W., Greenstein, L. A., White, R. M., Lee, L., Rechler, M. M. & Nissley, S. P. (1987) Type II insulin-like growth factor receptor is present in rat serum. *Proceedings of the National Academy of Sciences U.S.A.* **84** : 7720-7724.

King, R., Wells, J. R. E., Krieg, P., Snoswell, M., Brazier, J., Bagley, C. J., Wallace, J. C., Ballard, F. J., Ross, M. & Francis, G. L. (1992) Production and characterisation of recombinant insulin-like growth factor-I (IGF-I) and potent analogues of IGF-I, with Gly or Arg substituted for Glu^{3}, following their expression in *Escherichia coli* as fusion proteins. *Journal of Molecular Endocrinology* **8** : 29-41.

Kirschner, B. S. & Sutton, M. M. (1986) Somatomedin-C levels in growth-impaired children and adolescents with chronic inflammatory bowel disease. *Gastroenterology* **91** : 830-836.

Klapper, D. G., Svoboda, M. E. & Van Wyk, J. J. (1983) Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. *Endocrinology* **112** : 2215-2217.

Koldovsky, O. & Thornburg, W. (1987) Hormones in milk. *Journal of Pediatric Gastroenterology and Nutrition* **6** : 172-196.

Koldovsky, O., Philipps, A., Rao, R. K. & Schaudies, P. (1992) Possible role of milk-borne peptide growth factors for the breast-fed infant. In: *Regulatory Gut Peptides in Paediatric Gastroenterology and Nutrition.* Frontiers in Gastrointestinal Research Vol. 21 (Heinz-Erian, P., Deutsch, J., Granditsch, G. eds.), pp. 150-169, Karger, Basel.

Korman, I. Y., Termanini, B., Harmon, J. W., Finan, T. M. & Nardi, R .V. (1989) Insulin-like growth factor receptors in human gastrointestinal tract. *Gastroenterology* **96** : A269.

Korner, C., Nurnberg, B., Uhde, M. & Braulke, T. (1995) Mannose 6-phosphate/insulin-like growth factor II receptor fails to interact with G-proteins. Analysis of mutant cytoplasmic receptor domains. *The Journal of Biological Chemistry* **270** : 287-295.

Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annual Review of Biochemistry* **61** : 307-330.

Laburthe, M., Rouyer-Fessard, C. & Gammeltoft, S. (1988) Receptors for insulin-like growth factors I and II in rat gastrointestinal epithelium. *American Journal of Physiology* **254** : G457-G462.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227** : 680-685.

Langer, R. & Moses, M. (1991) Biocompatible controlled release polymers for delivery of polypeptides and growth factors. *Journal of Cellular Biochemistry* **45** : 340-345.

Langer, R., Siegel, R., Brown, L., Leong, K., Kost, J. & Edelman, E. (1985) Controlled release and magnetically modulated systems for macromolecular drugs. *Annals of the New York Academy of Sciences* **446** : 1-13. J.

Langford, K. S. & Miell, J. P. (1993) The insulin-like growth factor-I/binding protein axis: physiology, pathophysiology and therapeutic manipulation. *European Journal of Clinical Investigation* **23** : 503-516.

Laron, Z. (1993) Somatomedin-1 (insulin-like growth factor-I) in clinical use. Facts and potential. *Drugs* **45** : 1-8.

Laskowski, M., Haessler, H. A., Miech, R. P., Peanasky, R. J. & Laskowski, O. (1958) Effect of trypsin inhibitor on passage of insulin across the intestinal barrier. *Science* **127** : 1115-1116.

Leach, A. A. (1961) Chemical composition of gastric juice. In: *Biochemists' Handbook. Compiled by one hundred and seventy one contributors* (Long, C., ed.), pp. 911-914, E. & F. N. Sporn Ltd., London.

Lemmey, A. B., Martin, A. A., Read, L. C., Tomas, F. M., Owens, P. C. & Ballard, F. J. (1991) IGF-I and the truncated analogue des-(1-3)IGF-I enhance growth in rats after gut resection. *American Journal of Physiology* **260** : E213-219.

Lemmey, A. B., Ballard, F. J., Martin, A. A., Tomas, F. M., Howarth, G. S. & Read, L. C. (1994) Treatment with IGF-I peptides improves function of the remnant gut following small bowel resection in rats. *Growth Factors* **10** : 243-252.

Leof, E. B., Wharton, W., Van Wyk, J. J. & Pledger, W. J., (1982) Epidermal growth factor (EGF) and somatomedin-C regulate G1 progression in competent BALB/C-3T3 cells. *Experimental Cell Research* **141** :107-115.

LeRoith, D. & Roberts, C. R. Jr. (1991) At the cutting edge. Insulin-like growth factor I (IGF-I): a molecular basis for endocrine versus local action. *Molecular and Cellular Endocrinology* **77** : C57-C61.

Levine, G. M. (1991) Regulation of intestinal mucosal growth. In: *Growth of the Gastrointestinal Tract: Gastrointestinal Hormones and Growth Factors* (Morisset, J. & Soloman, T. E. eds.), pp. 175-189, CRC Press, Boca Raton.

Lindberg, T (1979) Protease inhibitors in human milk. *Pediatric Research* **13** : 969-972.

Lowe, W. L. Jr. (1991) Biological actions of the insulin-like growth factors. In: *Insulin-like Growth Factors: Molecular and Cellular Aspects* (LeRoith, D. ed.), pp. 49-85, CRC Press, Boca Raton.

Lowe, W. L. Jr., Roberts, C. T. Jr., Lasky, S. R. & LeRoith, D. (1987) Differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. *Proceedings of the National Academy of Sciences U.S.A.* **84 :** 8946-8950.

Lowe, W. L. Jr., Lasky, S. R., LeRoith, D. & Roberts, C. T. (1988) Distribution and regulation of rat insulin-like growth factor I messenger ribonucleic acids encoding alternative carboxyterminal E-peptides: evidence for differential processing and regulation in liver. *Molecular Endocrinology* **2** : 528-535.

Lowe, W. L., Adams, M., Werner, H., Roberts, C. T. & LeRoith, D. (1989) Regulation by fasting of rat insulin-like growth factor I and its receptor. Effects on gene expression and binding. *The Journal of Clinical Investigation* **84** : 619-626.

Lowman, H. B. & Wells, J. A. (1993) Affinity maturation of human growth hormone by monovalent phage display. *Journal of Molecular Biology* **243** : 564-578.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* **193** : 265-275.

Lu, L., Stanley, B. A. & Pegg, A. E. (1991) Identification of residues in ornithine decarboxylase essential for enzymatic activity and for rapid protein turnover. *Biochemical Journal* **277** : 671-675.

Lund, P. K. (1994) Insulin-like growth factors. In: *Gut peptides: Biochemistry and Physiology* (Walsh, J. H. & Dockray, G. J. ed.), pp. 587-613, Raven Press Ltd., New York.

Lund, P. K., Moats-Staats, B. M., Hynes, M. A., Simmons, J. G., Jansen, M., D'Ercole, A. J & Van Wyk, J. J. (1986) Somatomedin-C/insulin-like growth factor-I and insulin-like growth factor-II mRNAs in rat fetal and adult tissues. *The Journal of Biological Chemistry* **261** : 14539-14544.

Lynch, S. E., Colvin, R. B., Antoniades, H. N. (1989) Growth factors in wound healing. Single and synergistic effects on partial thickness porcine skin wounds. *Journal of Clinical Investigation* **84** : 640-646.

MacDonald, R. G., Tepper, M. A., Clairmont, K. B., Perregaux, S. B. & Czech, M. P. (1989) Serum form of the rat insulin-like growth factor II/mannose-6-phosphate receptors is truncated in the carboxyl-terminal domain. *The Journal of Biological Chemistry* **264** : 3256-3264.

McCusker, R. H. & Clemmons, D. R. (1992) The insulin-like growth factor binding proteins: structure and biological functions. In: *The Insulin-like Growth Factors. Structure and Biological Functions* (Schofield, P. N. ed.), pp. 110-150, Oxford University Press, Oxford.

McKinnon, P., Ross, M., Wells, J. R. E., Ballard, F. J. & Francis, G. L. (1991) Expression, purification and characterization of secreted recombinant human insulin-like growth factor-I (IGF-I) and the potent variant des(1-3)IGF-I in chinese hamster ovary cells. *Journal of Molecular Endocrinology* **6** : 231-239.

McMorris, F. A., Smith, T. M., Desalvo, S. & Furlanetto, R. W. (1986) Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proceedings of the National Academy of Sciences U.S.A.* **83 :** 822-826.

Maly, P. & Lüthi, C. (1988) The binding sites of insulin-like growth factor I (IGF-I) to type I IGF receptor and to a monoclonal antibody. Mapping by chemical modification of tyrosine residues. *The Journal of Biological Chemistry* **263** : 7.68-7072.

Marquardt, H., Todaro, G. J., Henderson, L. E. & Oroszlan, S. (1981) Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. Homology with human insulin-like growth factor II. *The Journal of Biological Chemistry* **256**: 6859-6865.

Marshall, G. R. (1993) A hierarchical approach to peptidomimetic design. *Tetrahedron* **49** : 3547-3558.

Massague, J. & Czech, M. P. (1982) The subunit structure of two distinct receptors for IGF-I and II and their relationship to the insulin receptor. *The Journal of Biological Chemistry* **257** : 5038-5045.

Mathews, L. S., Norstedt, G. & Palmiter, R. D. (1986) Regulation of insulin-like growth factor I gene expression by growth hormone. *Proceedings of the National Academy of Sciences U.S.A.* **83** : 9343-9347.

Mathews, L. S., Hammer, R. E., Behringer, R. R., D'Ercole, A. J., Bell, G. I., Brinster, R. L. & Palmiter, R. D. (1988) Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* **123** : 2827-2833.

Merimee, T. J., Grant, M. & Tyson, J. E. (1984). Insulin-like growth factors in amniotic fluid. *Journal of Clinical Endocrinology and Metabolism* **59** : 754-755.

Miell, J. P., Taylor, A. M., Jones, J., Buchanan, C. R., Rennie, J., Sherwood, R., Leicester, R. & Ross, R. J. M. (1992) Administration of human recombinant insulin-like growth factor-I to patients following major gastrointestinal surgery. *Clinical Endocrinology* **37** : 542-551.

Moller, D. E. & Flier, J. S. (1991) Insulin-resistance-mechanisms, syndromes, and implications. *New England Journal of Medicine* **325** : 938-948.

Morgan, D. O., Edam, J. C., Standring, D. N., Fried, V. A., Smith, M. C., Roth, R. A. & Rutter, W. J. (1987) Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* **329** : 301-307.

Morgan, J. M., Saris, S. D., Capuzzi, D. M., Good, W. R., John, V. A., Guler, H-P., Seaman, J. & Piraino, A. J. (1993) Hypoglycemic and insulin response to a continuous intravenous infusion of CGP-35126 recombinant human insulin-like growth factor-I (rhIGF-I) in healthy males. *The Journal of Clinical Pharmacology* **33** : 366-372.

Moss, J. A., Francis, G. L., Ross, M., Wallace, J. C. & Ballard, F. J. (1991) Insulin-like growth factor (IGF)-I and IGF-II binding to an IGF binding protein. An investigation using chemical modification of tyrosine residues as a structural probe for the sites of interactions. *The Journal of Biological Chemistry* **266** : 909-914.

Moxham, C. P., Duronio, V. & Jacobs, S. (1989) Insulin-like growth factor 1 receptor beta-subunit heterogeneity. Evidence for hybrid tetramers composed of insulin-like growth factor 1 and insulin receptor heterodimers. *The Journal of Biological Chemistry* **264** : 13238-13244.

Mulvihill, S. J., Stone, M. M., Debas, H. T. & Fonkalsrud, E. W. (1985) The role of amniotic fluid in fetal nutrition. *Journal of Pediatric Surgery* **20** : 668-672.

Murayama, Y., Okamoto, T., Ogata, E., Asano, T., Iiri, T., Katada, T., Ui, M., Grubb, J. H., Sly, W. S. & Nishimoto, I. (1990) Distinctive regulation of the functional linkage between the human cation-independent mannose 6-phosphate receptor and GTP-binding proteins by insulin-like growth factor II and mannose 6-phosphate. *The Journal of Biological Chemistry* **265** : 17456-17462.

Murphy, L. J., Bell, G. I & Friesen, H. G. (1987) Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. *Endocrinology* **120** : 1279-1282.

Murray-Rust, J., McLeod, A. N., Blundell, T. L. & Wood, S. P. (1992) Structure and evolution of insulins: implications for receptor binding. *BioEssays* **14** : 325-331.

Nagaoka, I., Trapnell, B. C. & Crystal, R. G. (1990) Regulation of insulin-like growth factor I gene expression in the human macrophage-like cell line U937. *Journal of Clinical Investigation* **85** : 448-455.

Nishimoto, I., Murayama, Y., Katada, T., Ui, M. & Ogata, E. (1989) Possible direct linkage of insulin-like growth factor-II receptor with guanine nucleotide-binding proteins. *The Journal of Biological Chemistry* **264** : 14029-14038.

Nissley, P. & Lopaczynski, W. (1991) Mini Review: insulin-like growth factor receptors. *Growth Factors* **5** : 29-43.

Nissley, S. P., Kiess, W. & Sklar, M. M. (1991) The insulin-like growth factor-II/mannose-6-phosphate receptor. In: *Insulin-like Growth Factors: Molecular and Cellular Aspects* (LeRoith, D. ed.), pp. 111-150, CRC Press, Boca Raton.

Ogasawara, M., Karey, K. P., Marquardt, H. & Sirbasku, D. A. (1989) Identification and purification of truncated insulin-like growth factor I from porcine uterus. Evidence for high biological potency. *Biochemistry* **28** : 2710-2720.

Oguchi, S., Walker, W. A. & Sanderson, I. R. (1993) Insulin-like growth factor binding protein profile secreted by intestinal and epithelial cells varies with polarity. *Biochemical and Biophysical Research Communications* **196** : 789-793.

Oh, Y., Beukers, M. W., Pham, H. M., Smanik, P. A., Smith, M. C. & Rosenfeld, R. G. (1991) Altered affinity of insulin-like growth factor II (IGF-II) for receptors and IGF-binding proteins, resulting from limited modifications of the IGF-II molecule. *Biochemical Journal* **278** : 249-254.

Oh, Y., Müller, H. L., Lee, D-Y., Fielder, P. J. & Rosenfeld, R. G. (1993) Characterization of the affinities of insulin-like growth factor (IGF) binding proteins 1-4 for IGF-I, IGF-II, IGF-I/insulin hybrid and IGF-I analogs. *Endocrinology* **132** : 1337-1344.

Oka, Y., Rozek, L. M. & Czech, M. P. (1985) Direct demonstration of rapid insulin-like growth factor II receptor internalization and recycling in rat adipocytes. Insulin stimulates ^{125}I-insulin-like growth factor II degradation by modulating the IGF-II receptor recycling process. *The Journal of Biological Chemistry* **260** : 9435-9442.

Olanrewaju, H., Patel, L. & Seidel, E. R. (1992) Trophic action of local intraileal infusion of insulin-like growth factor I: polyamine dependence. *American Journal of Physiology* **263** : E282-E286.

Örd, T., Kolmer, M., Villems, R. & Saarma, M. (1990) Structure of the human genomic region homologous to the bovine prochymosin-encoding gene. *Gene* **91** : 241-246.

Oth, M., Franz, M., Timmermans, J & Möes, A. (1992) The bilayer floating capsules: a stomach-directed drug delivery system for misoprostol. *Pharmaceutical Research* **9** : 298-302.

Owens, P. C., Waters, M. J., Thorburn, G. D. & Brinsmead, M. W. (1985) Insulin-like growth factor receptor in fetal lamb liver: characterization and developmental changes. *Endocrinology* **117** : 982-990.

Park, J. H. Y., Vanderhoof, J. A., Blackwood, D. & MacDonald, R. G. (1990) Characterization of type I and type II insulin-like growth factor receptors in an intestinal epithelial cell line. *Endocrinology* **126** : 2998-3005.

Park, J. H. Y., Vanderhoof, J. A., Mohammadpour, H. & MacDonald, R. G. (1992) Production of insulin-like growth factor II (IGF-II) and IGF binding protein-2 (IGFBP-2) by rat intestinal epithelial cells. *Gastroenterology* **102** : A572.

Pell, J. M. & Bates, P. C. (1992) Differential actions of growth hormone and insulin-like growth factor-I on tissue protein metabolism in dwarf mice. *Endocrinology* **130** : 1942-1950.

Peters, M. A., Lau, E. P., Snitman, D. L., Van Wyk, J. J., Underwood, L. E., Russell, W. E. & Svoboda, M. E. (1985) Expression of a biologically active analogue of somatomedin-C/insulin-like growth factor I. *Gene* **35** : 83-89.

Philipps, A. F., Persson, B., Hall, K., Lake, M., Skottner, A., Sanengen, T. & Sara, V. (1988) The effects of biosynthetic insulin-like growth factor-I supplementation on somatic growth, maturation and erythropoiesis on the neonatal rat. *Pediatric Research* **23** : 298-305.

Philipps, A. F., Rao, R., McCracken, D. & Koldovsky, O. (1990) Presence of insulin-like growth factor (IGF-I) in rat milk and the absorption of IGF-I by the suckling rat. *Pediatric Research* **27** : 49A.

Philipps, A., Rao, R. K., McCracken, D. & Koldovsky, O. (1992) Fate of orogastrically administered insulin-like growth factors I and II (IGF-I and -II) to suckling rats. *Gastroenterology* **102** : A572.

Pierson, R. W. Jr. & Temin, H. M. (1972) The partial purification from calf serum of a fraction with multiplication-stimulating action for chicken fibroblasts in cell culture and with non-suppressible insulin-like activity. *Journal of Cellular Physiology* **79** : 319-330.

Pillion, D. J., Haskell, J. F., Atchinson, J. A., Ganapathy, V. & Leibach, F. H. (1989) Receptors for IGF-I, but not for IGF-II, on proximal colon epithelial cell apical membranes. *American Journal of Physiology* **257** :E27-34.

Powell, D., Lee, P., Change, D., Liu, F. & Hintz, R. (1987) Antiserum developed for the E peptide region of insulin-like growth factor IA prohormone recognizes a serum protein by both immunoblot and radioimmunoassay. *Journal of Clinical Endocrinology and Metabolism* **65** :868-875.

Powers, J. C., Harley, A. D. & Myers, D. V. (1977) Subsite specificity of porcine pepsin. *Advances in Experimental Medicine and Biology* **95** : 141-157.

Pritchard, J. A. (1966) Fetal swallowing and amniotic fluid volume. *Obstetrics and Gynecology* **28** : 606.

Quin, J. D., Fisher, B. M., Paterson, K .R., Inoue, A., Beastall, G. H. & MacCuish, A. C. (1990) Acute response to recombinant insulin-like growth factor I in a patient with Mendenhall's syndrome. *New England Journal of Medicine* **323** : 1425-1426.

Ranade, V. V. (1991) Drug Delivery Systems 5A. Oral drug delivery. *Journal of Clinical Pharmacology* **31** : 2-16.

Rao, R. K. (1991) Biologically active peptides in the gastrointestinal lumen. *Life Sciences* **48** : 1685-1704.

Rao, R. K., Lam, K., Philipps, A. F., Williams, C., Lake, M. & Koldovsky, O. (1993) Presence of multiple forms of peptidase inhibitors in rat milk. *Journal of Pediatric Gastroenterology and Nutrition* **17** : 414-420.

Read, L. C., Ballard, F. J., Francis, G. L., Baxter, R. C., Bagley, C. J. & Wallace, J. C. (1986) Comparative binding of bovine, human and rat insulin-like growth factors to membrane receptors and to antibodies against human insulin like growth factor-I. *Biochemical Journal* **223** : 215-221.

Read, L. C., Lemmey, A. B., Howarth, G. S., Martin, A. A., Tomas, F. M. & Ballard, F. J. (1991) The gastrointestinal tact is one of the most responsive target tissues for IGF-I and its potent analogs. In: *Modern Concepts of Insulin-like Growth Factors* (Spencer, E. M. ed.), pp. 225-234, Elsevier, New York.

Read, L. C., Tomas, F. M., Howarth, G. S., Martin, A. A., Edson, K. J., Gillespie, C. M., Owens, P. C. & Ballard, F. J. (1992a) Insulin-like growth factor-I and its N-terminal modified analogues induce marked gut growth in dexamethasone-treated rats. *Journal of Endocrinology* **133** : 421-432.

Read, L. C., Howarth, G. S., Lemmey, A. B., Steeb, C-B., Trahair, J., Tomas, F. M. & Ballard, F. J. (1992b) The gastrointestinal tract: a most sensitive target for IGF-I. *Proceedings of the Nutrition Society of New Zealand* **17** : 136-142.

Read, L., Howarth, G., Cool, J., Steeb, C., Lemmey, A. & Penning, K. (1994) IGFs and gut disease. Proceedings of the Diagnostic and Scientific Laboratory Meeting, Adelaide.

Rechler, M. W. (1993) Insulin-like growth factor binding proteins. *Vitamins and Hormones* **47** : 1-114.

Rechler, M. M. & Nissley, S. P. (1985) The nature and regulation of the receptors for insulin-like growth factors. *Annual Review of Physiology* **47** : 425-442.

Regnstrom, K., Aberg, A., Ormo, M., Sahlin, M. & Sjoberg, B. M. (1994) The conserved serine 211 is essential for reduction of the dinuclear iron center in protein R2 of *Escherichia coli* ribonucleotide reductase. *The Journal of Biological Chemistry* **269** : 6355-6361.

Rinderknecht, E. & Humbel, R. E. (1976) Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: isolation, chemical characterization, and some biological properties of form I and II. *Proceedings of the National Academy of Sciences U.S.A.* **73** : 2365-2369.

Rinderknecht, E. & Humbel, R. E. (1978a) The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *The Journal of Biological Chemistry* **253** : 2769-2776.

Rinderknecht, E. & Humbel, R. E. (1978b) Primary structure of human insulin-like growth factor II. *FEBS Letters* **89** : 283-286.

Robberecht, P., Deschodt-Lanckman, M., Camus, J., Bruylands, J. & Christophe, J. (1971) Rat pancreatic hydrolases from birth to weaning and dietary adaptation after weaning. *American Journal of Physiology* **221** : 376-381.

Rom, W. N., Basset, P., Fells, G. A., Nukiwa, T., Trapnell, B. C. & Crystal R. G. (1988) Alveolar macrophages release an insulin-like growth factor I-type molecule. *Journal of Clinical Investigation* **82** : 1685-1693.

Romanus, J. A., Yang, Y. W. H., Adams, S. O., Sofair, A. N., Tseng, L. Y. H., Nissley, S. P. & Rechler, M. M. (1988) Synthesis of insulin-like growth factor II (IGF-II) in fetal rat tissues: translation of IGF-II ribonucleic acid and processing of pre-pro-IGF-II. *Endocrinology* **122** : 709-716.

Rosenbloom, A. L. Guevara-Aguirre, J., Vaccerello, M., Rosenfeld, R. G., Fielder, P. J. & Diamond, F. B. (1991) Growth hormone receptor deficiency (Laron syndrome). In: *Modern Concepts of Insulin-like Growth Factors* (Spencer, E. M. ed.), pp. 49-79, Elsevier, New York.

Ross, M., Francis, G. L., Szabo, L., Wallace, J. C. & Ballard, F. J. (1989) Insulin-like growth factor (IGF)-binding proteins inhibit the biological activities of IGF-I and IGF-II, but not des(1-3)IGF-I. *Biochemical Journal* **258** : 267-272.

Rossi, T. M., Lee, P. C. & Lebenthal, E. (1983) Effect of feeding regimens on the functional recovery of pancreatic enzymes in postnatally malnourished weanling rats. *Pediatric Research* **17** : 806-809.

Roth, B. V., Bürgisser, D. M., Lüthi, C. & Humbel, R. E. (1991) Mutants of human insulin-like growth factor II: expression and characterization of analogues with a substitution of Tyr^{27} and/or deletion of residues 62-67. *Biochemical and Biophysical Research Communications* **181** : 907-914.

Rotwein, P. (1986) Two insulin-like growth factor-I messenger RNAs are expressed in human liver. *Proceedings of the National Academy of Sciences U.S.A.* **83** : 77-81.

Rotwein, P. (1991) Structure, evolution, expression and regulation of insulin-like growth factors I and II. *Growth Factors* **5** : 3-18.

Rotwein, P., Folz, R. J. & Gordon, J. I. (1987a) Biosynthesis of human insulin-like growth factor I (IGF-I). *The Journal of Biological Chemistry* **262** : 11807-11812.

Rotwein, P., Pollock, K. M., Watson, M. & Milbrandt, J. D. (1987b) Insulin-like growth factor gene expression during rat embryonic development. *Endocrinology* **121** : 1241-1244.

Rouyer-Fessard, C., Gammeltoft, S. & Laburthe, M. C. (1990) Expression of two types of receptor for insulinlike growth factors in human colonic epithelium. *Gastroenterology* **98** : 703-708.

Ryan, J. & Costiga, D. C. (1993) Determination of the histological distribution of insulin like growth factor 1 receptors in the rat gut. *Gut* **34** : 1693-1697.

Ryle, A. (1970) The porcine pepsins and pepsinogens. *Methods in Enzymology* **19** : 316-336.

Saffran, M., Kumar, G. S., Savariar, C., Burnham, J. C. Williams, F. & Neckers, D. C. (1986) A new approach to the oral administration of insulin and other peptide drugs. *Science* **233** : 1081-1084.

Said, S. I. (1992) Biological and physiological actions of regulatory gut peptides in the gastrointestinal tract. In: *Regulatory Gut Peptides in Paediatric Gastroenterology and Nutrition.* Frontiers in Gastrointestinal Research Vol. 21 (Heinz-Erian, P., Deutsch, J., Granditsch, G. eds.), pp. 49-57, Karger, Basel.

Sakano, K., Enjoh, T., Numata, F., Fujiwara, H., Marumoto, Y., Higashihashi, N., Sato. Y., Perdue, J. F. & Fujita-Yamaguchi, Y. (1991) The design, expression and characterization of human insulin-like growth factor II (IGF-II) mutants specific for either the IGF-II/cation-independent mannose 6-phosphate receptor or IGF-I receptor. *The Journal of Biological Chemistry* **266** : 20626-20635.

Salmon, W. D. Jr & Daughaday, W. H. (1957) A hormonally controlled serum factor which stimulates sulphate incorporation by cartilage *in vitro*. *The Journal of Laboratory and Clinical Medicine* **49** : 825-836.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular cloning, a laboratory manual. 2nd Ed., (Nolan, C., Ford, N. & Ferguson, M. eds.) Cold Spring Harbour Press, New York.

Sanger, F., Nicklen, S. & Coulsen, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences U.S.A.* **74** : 5463-5467.

Sara, V. R. & Hall, K. (1990) Insulin-like growth factors and their binding proteins. *Physiological Reviews* **70** : 591-614.

Sara, V. R., Carlsson-Skwirut, C., Bergman, T., Jörnvall, H., Roberts, P. J., Crawford, M., Nilsson Hakansson, L., Civalero, I & Nordberg, A. (1989) Identification of Gly-Pro-Glu (GPE), the aminoterminal tripeptide of insulin-like growth factor I which is truncated in brain, as a novel neuroactive peptide. *Biochemical and Biophysical Research Communications* **165** : 766-771.

Sato, A., Nishimura, S., Ohkubo, T., Kyogoku, Y., Koyama, S., Kobayashi, M., Yasuda, T. & Kobayashi, Y. (1992) ^{1}H-NMR assignment and secondary structure of human insulin-like growth factor (IGF-I) in solution. *Journal of Biochemistry* **111** : 529-536.

Sato, A., Nishimura, S., Ohkubo, T., Kyogoku, Y., Koyama, S., Kobayashi, M., Yasuda, T. & Kobayashi, Y. (1993) Three-dimensional structure of human insulin-like growth factor-I (IGF-I) determined by ^{1}H-NMR and distance geometry. *International Journal of Peptide and Protein Research* **41** : 433-440.

Satoh, H., Shino, A., Inatomi, N., Nagaya, H., Sato, F. & Szabo, S. (1991) Effects of rbbFGF mutein CS23 (TGP-580) on the healing of gastric ulcers induced by acetic acid in rats. *Gastroenterology* **100** : A155.

Schäffer, L., Larsen, U. D., Linde, S., Hejnæs, K. R. & Skriver, L. (1993) Characterization of the three ^{125}I-iodination isomers of human insulin-like growth factor I (IGF I). *Biochemica et Biophysica Acta* **1203** : 205-209.

Schechter, I. & Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochemical and Biophysical Research Communications* **27** : 157-162.

Scheiwiller, E., Guler, H. P., Merryweather, J., Scandella, C., Maerki, W., Zapf, J. & Froesch, E. R. (1986) Growth restoration of insulin-deficient diabetic rats by recombinant human insulin-like growth factor-I *Nature* **323** : 169-171.

Schmid, C., Steiner, T. & Froesch, E. R. (1983) Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF I and IGF II) in primary cultures of chicken embryonic cells. *FEBS Letters* **161** : 117-121.

Schmid, C., Steiner, T. & Froesch, E. R. (1984) Insulin-like growth factor I supports differentiation of cultured osteoblast-like cells. *FEBS Letters* **173** : 48-52.

Schober, D. A., Simmen, F. A., Hadsell, D. L. & Baumrucker, C. R. (1990) Perinatal expression of type 1 IGF receptors in porcine small intestine. *Endocrinology* **126** : 1125-1132.

Schoenle, E., Zapf, J., Hauri, C., Steiner, T & Froesch, E. R. (1985) Comparison of *in vivo* effects of insulin-like growth factors I and II and of growth hormone in hypophysectomized rats. *Acta Endocrinologica* **108** : 167-174.

Schoenle, E. J., Zenobi, P. D., Torresani, T., Werder, E. A., Zachmann, M. & Froesch, E. R. (1991) Recombinant human insulin-like growth factor (rhIGF I) reduces hyperglycaemia in patients with extreme insulin-resistance. *Diabetologia* **34** : 675-679.

Scott, J., Cowell, J., Robertson, M. E., Priestley, L. M., Wadey, R., Hopkins, B., Pritchard, J., Bell, G. I., Rall, L. B., Graham, C. F. & Knott, T. J. (1985) Insulin-like growth factor-II gene expression in Wilms' tumour and embryonic tissues. *Nature* **317** : 260-262.

Seidel, E. R., Ginty, D. D. & Marcuard, S. P. (1989) Insulin-like growth factor-I in human exocrine secretions. *Gastroenterology* **96** : A459.

Seidel, E. R., Chaurasia, O. P. & Groblewski, G. E. (1992) Intraluminal IGF-I and stimulation of gastrointestinal mucosal growth. Proceedings of the 2nd International Symposia on Insulin-like Growth Factors, San Franciso, California, Jan 12-16th, Abstract B23.

Seno, M., Sasada, R., Iwane, M., Sudo, K., Kurokawa, T., Ito, K. & Igarashi, K. (1988) Stabilizing basic fibrobast growth factor using protein engineering. *Biochemical and Biophysical Research Communications* **151** : 701-708.

Shamblott, M. J. & Chen, T. T. (1992) Identification of a second insulin-like growth factor in a fish species. *Proceedings of the National Academy of Sciences U.S.A.* **89** : 8913-8917.

Sherwin, R. S., Borg, W. P. & Boulware, S. D. (1994) Metabolic effects of insulin-like growth factor I in normal humans. *Hormone Research* **41** (Suppl. 2) : 97-102.

Shichiri, M., Okada, A., Kikkawa, R., Kawamori, R. & Shigeta, Y. (1971) β-naphthyl-azo-polystyrene-insulin as a means of protecting insulin molecule from digestive enzymes. *Biochemical and Biophysical Research Communications* **44** : 51-56.

Shimasaki, S., Shimonaka, M., Zhang, H. P. & Ling, N. (1991) Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. *The Journal of Biological Chemistry* **266** : 10646-10653.

Shimatsu, A & Rotwein, P. (1987) Mosaic evolution of the insulin-like growth factors. Organization, sequence and expression of the rat insulin-like growth factor I gene. *The Journal of Biological Chemistry* **262** : 7894-7900.

Singh, P. & Rubin, N. (1993) Insulinlike growth factors and binding proteins in colon cancer. *Gastroenterology* **105** : 1218-1237.

Skottner, A., Clark, R. G., Robinson, I. C. A. F. & Fryklund, L. (1987) Recombinant human insulin-like growth factor: testing the somatomedian hypothesis in hypophysectomized rats. *Journal of Endocrinology* **112** : 123-132.

Skottner, A., Clark, R. G., Fryklund, L. & Robinson, I. C. A. F. (1989) Growth responses in mutant dwarf rat to human growth hormone and recombinant human insulin-like growth factor. *Endocrinology* **124** : 2519-2526.

Skottner, A., Arrhenius-Nyberg, V., Kanje, M. & Fryklund, L. (1990) Anabolic and tissue repair function of recombinant insulin-like growth factor I. *Acta Paediatrica Scandinavica Supplement* **367** : 63-66.

Slieker, L. J., Brooke, G. S., Chance, R. E., Fan, L., Hoffmann, J. A., Howey, D. C., Long, H. B., Mayer, J., Shields, J. E., Sundell, K. L. & DiMarchi, R. D. (1994) Insulin and IGF-I analogs: novel approaches to improved insulin pharmacokinetics. In: *Current Directions in Insulin-like Growth Factor Research* (LeRoith, D. & Raizada, M. K. eds.), pp. 25-32, Plenum Press, New York.

Smith, M. C., Cook, J. A., Furman, T. C., & Occolonwitz, J. L. (1989) Structure and activity dependence of recombinant human insulin-like growth factor II on disulfide bond pairing. *The Journal of Biological Chemistry* **264** : 9314-9321.

Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C. & Rubin, C. S. (1988) Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *The Journal of Biological Chemistry* **263** : 9402-9408.

Soares, M. B., Ishii, D. N. & Efstratiadis, A. (1985) Developmental and tissue-specific expression of a family of transcripts related to rat insulin-like growth factor II mRNA. *Nucleic Acids Research* **13** : 1119-1133.

Soos, M. A. & Siddle, K. (1989) Immunological relationships between receptors for insulin and insulin-like growth factor I. Evidence for structural heterogeneity of insulin-like growth factor I receptors involving hybrids with insulin receptors. *Biochemical Journal* **263** : 553-563.

Spencer, E. M., Skover, G. & Hunt, T. K. (1988) Somatomedins: do they play a pivotal role in wound healing? *Progress in Clinical and Biological Research* **266** : 103-116.

Sporn, M. B. & Roberts, A. B. (1988) Peptide growth factors are multifunctional. *Nature* **332** : 217-219.

Steeb, C-B., Trahair, J. F., Tomas, F. M. & Read, L. C. (1994) Prolonged administration of IGF peptides enhances growth of gastrointestinal tissues in normal rats. *American Journal of Physiology* **266** : G1090-G1098.

Steenfoe, H., Spencer, E. M., & Hunt, T. K., (1989) Insulin-like growth factor I has a major role in wound healing. *Surgical Forum* **40** : 68-70.

Stempien, M. M., Fong, N. M., Rall, L. B. & Bell, G. I. (1986) Sequence of a placental cDNA encoding the mouse insulin-like growth factor II precursor. *DNA* **5** : 357-361.

Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., Van Wyk, J. J. & Pledger, W. J. (1979) Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proceedings of the National Academy of Sciences U.S.A.* **76** : 1279-1283.

Strock, L., Singh, H., Abdullah, A., Miller, J. A. & Herndon, D. N. (1990) The effect of insulin-like growth factor I on postburn hypermetabolism *Surgery* **108** : 161-164.

Suh, D. Y., Hunt, T. K. & Spencer, E. M. (1992) Insulin-like growth factor-I reverses the impairment of wound healing induced by corticosteroids in rats. *Endocrinology* **181** : 2399-2403.

Sussenbach, J. S. (1989) The gene structure of the insulin-like growth factor family. *Progress in Growth Factor Research* **1** : 33-48.

Szabo, L., Mottershead, D. G., Ballard, F. J. & Wallace, J. C. (1988) The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-I for binding. *Biochemical and Biophysical Research Communications* **151** : 207-214.

Szabo, S., Folkman, J., Vatiay, P., Morales, R. E., Pinkus, G. S. & Kato, K. (1994) Accelerated healing of duodenal ulcers by oral administration of a mutein of basic fibroblast growth factor in rats. *Gastroenterology* **106** : 1106-1111.

Szecsi, P. B. (1992) The aspartic proteases. *Scandinavian Journal of Clinical and Laboratory Investigation* **52** (Suppl. 210) : 5-22.

Tang, J. (1970) Gastricsin and Pepsin. *Methods in Enzymology* **19** : 406-421.

Taylor, S. I., Kadowaki, T., Kadowaki, H., Accili, D., Cama, A. & McKeon, C. (1990) Mutations in insulin-receptor gene in insulin-resistant patients. *Diabetes Care* **13** : 257-279.

Termanini, B., Nardi, R. V., Finan, T. M., Parikh, I. & Korman, L. Y. (1990) Insulinlike growth factor I receptors in rabbit gastrointestinal tract. Characterization and autoradiographic localization. *Gastroenterology* **99** : 51-60.

Thissen, J-P., Ketelslegers, J-M. & Underwood, L. E. (1994) Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews* **15** : 80-101.

Tokunaga, A., Hunt, T. K. & Spencer, E. M. (1990) Insulin-like growth factor binding protein-3 is present in the alpha granules of platelets. Proceedings of the 72nd Annual Meeting of the Endocrine Society, Washington DC, Abstract p71.

Tomas, F. M., Knowles, S. E., Owens, P. C., Chandler, C. S., Francis, G. L., Read, L. C. & Ballard, F. J. (1992) Insulin-like growth factor-I (IGF-I) and especially IGF-I variants are anabolic in dexamethasone treated rats. *Biochemical Journal* **282** : 91-97.

Torres, A. M., Forbes, B. E., Aplin, S. E., Wallace, J. C., Francis, G. L. & Norton, R. S. (1995) Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. *Journal of Molecular Biology* **248** : 385-401.

Treadway, J. L., Morrison, B. D., Frattali, A. L. & Pessin, J. E. (1991) Transmembrane signalling of insulin/IGF-I hybrid receptors. In: *Modern Concepts of Insulin-like Growth Factors* (Spencer, E. M. ed.), pp. 449-489, Elsevier Science Publishing Co. Inc., London.

Trout, G. E. & Fruton, J. S. (1969) The side-chain specificity of pepsin. *Biochemistry* **8** : 4183-4190.

Tseng, L. Y-H., Schwartz, G. P., Sheikh, M., Chen, Z. Z., Joshi, S., Wang, J-F., Nissley, S. P., Burke, G. T., Katsoyannis, P. G. & Rechler, M. M. (1987) Hybrid molecules containing the A-domain of insulin-like growth factor-I and the B-chain of insulin have increased mitogenic activity relative to insulin. *Biochemical and Biophysical Research Communications* **149** : 672-679.

Turkalj, I., Keller, U., Ninnis, R., Vosmeer, S. & Stauffacher, W. (1992) Effect of increasing doses of recombinant human insulin-like growth factor-I on glucose, lipid and leucine metabolism in man. *Journal of Clinical Endocrinology and Metabolism* **75** : 1186-1190.

Umezawa, H. (1976) Structures and activities of protease inhibitors of microbial origin. *Methods in Enzymology* **45** : 678-695.

Upton, F. Z., Francis, G. L., Ross, M., Wallace, J. C. & Ballard, F. J. (1992) Production and characterization of recombinant chicken insulin-like growth factor-I from *Escherichia coli*. *Journal of Molecular Endocrinology* **9** : 83-92.

Upton, Z., Francis, G. L., Kita, K., Wallace, J. C. & Ballard, F. J. (1995) Production and characterization of recombinant chicken insulin-like growth factor-II from *Escherichia coli*. *Journal of Molecular Endocrinology* **14** : 79-90.

Van Dijk, M. A., Van Schaik, F. M. A., Bootsma, H. J., Holthuizen, P. & Sussenbach, J. S. (1991) Initial characterisation of the four promoters of the human insulin-like growth factor II gene. *Molecular and Cellular Endocrinology* **81** : 81-94.

Vanderhoof, J. A., McCusker, R. H., Clark, R., Mohammadpour, H., Blackwood, D. J., Harty, R. F. & Park, J. H. Y. (1992) Truncated and native insulinlike growth factor I enhance mucosal adaptation after jejunoileal resection. *Gastroenterology* **102** : 1949-1956.

Van Obberghen-Schilling, E. & Pouyssegur, J., (1983) Mitogen-potentiating action and binding characteristics of insulin and insulin-like growth factors in chinese hamster fibroblasts. *Experimental Cell Research* **147** : 369-378.

Veldhuis, J. D., Rodgers, R. J. & Furlanetto, R. W. (1986) Synergistic actions of estradiol and the insulin-like growth factor somatomedin-C on swine ovarian (granulosa) cells. *Endocrinology* **119** : 530-538.

Visser, S., Van Rooijen, P. J., Schattenkerk, C. & Kerling, K. E. T. (1976) Peptide substrates for chymosin (renin). Kinetic studies with peptides of different chain length including parts of the sequence 101-112 of bovine κ- casein. *Biochemica et Biophysica Acta* **438** : 265-272.

Walker, J. L., Van Wyk, J. J. & Underwood, L. E. (1992) Stimulation of statural growth by recombinant insulin-like growth factor I in a child with growth hormone insensitivity syndrome (Laron type). *The Journal of Pediatrics* **121** : 641-646.

Walker, V. & Taylor, W. H. (1979) Pepsin 5 in gastric juice: Determination and relationship to the alkali-stable peptic activity. *Gut* **20** : 977-982.

Ward, A. & Ellis, C. J. (1992) The insulin-like growth factor genes. In: *The Insulin-like Growth Factors. Structure and Biological Functions* (Schofield, P. N. ed.), pp. 45-79, Oxford University Press, Oxford.

Werner, H., Woloschak, M., Stannard, B., Shen-Orr, Z., Roberts, C. T. Jr. & LeRoith, D. (1991) The insulin-like growth factor I receptor: molecular biology, heterogeneity and regulation. In: *Insulin-like Growth Factors: Molecular and Cellular Aspects* (LeRoith, D. ed.), pp. 17-4, CRC Press, Boca Raton.

Weström, B. R., Svedsen, J. & Karlsson, B. W. (1982) Protease inhibitor levels in porcine mammary secretions. *Biology of the Neonate* **42** : 185-194.

Wilton, P. (1992) Treatment with recombinant human insulin-like growth factor I of children with growth hormone receptor deficiency (Laron Syndrome). *Acta Paediatrica Scandinavica Supplement* **383** : 137-141.

Xian, C. J., Shoubridge, C. A. & Read, L. C. (1995) Degradation of IGF-I in the adult rat gastrointestinal tract is limited by a specific antiserum or the dietary protein casein. *Journal of Endocrinology* **146** : 215-225.

Yang, Y. W. H., Romanus, J. A., Liu, T. Y., Nissley, S. P. & Rechler, M. M. (1985) Biosynthesis of rat insulin-like growth factor II: I. Immunochemical demonstration of a ~20-kilodalton biosynthetic precursor of rat insulin-like growth factor II in metabolically labelled BRL-3A rat liver cells. *The Journal of Biological Chemistry* **260** : 2570-2577.

Yarden, Y. & Ullrich, A. (1988) Growth factor receptor tyrosine kinases. *Annual Review of Biochemistry* **57** : 443-478.

Young, G. P., Tarranto, T. M., Jonas, H. A., Cox, A. J., Hogg, A. & Werther, G. A. (1990) Insulin-like growth factors and the developing and mature rat small intestine: receptors and biological actions. *Digestion* **46** (Suppl. 2) : 240-252.

Young, S. C., Underwood, L. E., Celniker, A. & Clemmons, D. R. (1992) Effects of recombinant insulin-like growth factor-I (IGF-I) and growth hormone on serum IGF binding proteins in calorically restricted adults. *Journal of Clinical Endocrinology and Metabolism* **75** : 603-608.

Zapf, J., Froesch, E. R. & Humbel, R. E. (1981) The insulin-like growth factors of human serum: chemical and biological characterization and aspects of their possible physiological role. *Current Topics in Cellular Regulation* **19** : 257-309.

Zapf, J., Hauri, C., Waldvogel, M. & Froesch, E. R. (1986) Acute metabolic effects and half-lives of intravenously administered insulin-like growth factors I and II in normal and hypophysectomized rats. *The Journal of Clinical Investigation* **77** : 1768-1775.

Ziv, E., Lior, O. & Kidron, M. (1987) Absorption of protein via the intestinal wall. A quantitative model. *Biochemical Pharmacology* **36** : 1035-1039.

Zumstein, P. P., Lüthi, C. & Humbel, R. E. (1985) Amino acid sequences of a variant pro-form of insulin-like growth factor II. *Proceedings of the National Academy of Sciences U.S.A.* **82** : 3169-3172.

APPENDIX I

The residue exchange matrices from Bordo & Argos (1991).

Observed substitutions for (a) buried, (b) exposed and (c) all cases. The lower halves of the matrices give substitution counts for central residues with 90 % or greater similar environments, while the upper halves are for 70 % or greater similarity. When counts show a statistically meaningful (95 % or greater confidence) increase or decrease compared to the expected values for at least two similarity levels ranging from 100 % to 70 % in steps of 5 %, with the trend being consistent, a + or - sign is given to indicate preferred or avoided substitutions, respectively. In the exposed data, immunoglobulin, variable domains were not included.

An example from the matrices: Phe in an exposed position.

From matrix (b), a Phe to Tyr substitution is a preferred exchange which is statistically significant whereas a Ser substitution is an exchange to avoid. Other amino acid exchanges for Phe are not statistically significant, although Thr, Asn and Ile have more observed exchange counts than other amino acid. In environments of greater than 90 % similarity, Met, Leu and Thr each have a single exchange count while Tyr has a slighly higher count of three.

	G	A	S	C	T	P	D	V	N	L	I	Q	M	E	H	K	F	R	Y	W
G		23+	6	3	5	0	0	8-	0	4	3	1	1	0	0	0	1-	0	0	0
A	9		18+	4	13+	3	1	32+	3	14	8	3	2	1	0	0	5	1	2	0
S	1	5		1	16+	1	2	5	8	4	3	0	1	0	0	0	3	0	1	1
C	0	1	0		0	0	0	4	0	0	1	0	0	0	0	0	1	0	0	0
T	0	4	5	0		1	1	10	2	3	6	0	4	1	0	0	2	0	0	0
P	0	0	0	0	0		0	1	0	0	0	0	0	0	0	0	1	0	0	0
D	0	0	0	0	0	0		0	3+	0	0	0	0	0	0	0	0	0	0	0
V	1	2	1	1	1	0	0		0	34+	39+	0	11	0	3	0	6	0	3	0
N	0	0	1	0	1	0	1	0		1	0	2	1	0	0	0	1	0	1	0
L	0	1	0	0	0	0	0	3	0		19+	2	15+	0	1	0	13+	0	4	5
I	0	1	0	0	1	0	0	10	0	3		2	4	0	0	0	4	1	1	4
Q	0	0	0	0	0	0	0	0	0	0	0		1	1	0	0	1	0	1	1
M	0	0	0	0	1	0	0	1	1	2	1	0		1	1	0	3	0	1	1
E	0	0	0	0	0	0	0	0	0	0	0	1	0		0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	1	1	0	0
K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	1	0	0
F	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0		0	6+	1
R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0		1	0
Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0		4+
W	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	

(a)

	G	A	S	C	T	P	D	V	N	L	I	Q	M	E	H	K	F	R	Y	W
G		29+	33+	1	20	19	23	3	30+	1-	1-	7	1	14	4	23	1	7	5	1
A	8		53+	2	30+	19+	23	6	26	5	5	20	2	32+	7	34+	3	9	4-	2
S	10	17		1	80+	29	45	13	50	6-	4	34	2	25	14	35	2-	20	4-	0
C	0	0	0		2	1	0	0	0	2	1	0	0	1	0	0	0	0	0	0
T	2	9	21	0		20	20	17	34	14	10	30	5	25	7	38+	7	17	7-	2
P	3	5	6	0	3		21	6-	12	3-	2	11	1	19	8	21	0	6	1	1
D	2	2	9	0	1	5		7-	45+	6	4	17	1	42+	4	25-	2	5	2-	0
V	1	2	2	0	3	0	0		6	14+	14+	7	1	10	0	15	2	8	4	0
N	7	3	7	0	3	0	11	1		10	5	18	1	13	6	32	5	7	6-	2
L	0	1	1	0	1	0	1	2	2		9+	13	4	8	1	14	3	7	7	3
I	0	1	1	0	2	0	0	2	0	4		6	3	7	1	7	6	2	3	1
Q	0	3	4	0	6	1	4	1	3	1	0		5	23+	6	29+	1	11	3	0
M	0	0	1	0	0	0	0	0	0	1	1	1		1	1	3	2	1	0	0
E	3	6	5	0	4	4	10	1	2	1	1	3	0		3	30	3	6	5	0
H	2	1	0	0	1	1	0	0	1	0	0	2	0	0		9	2	1	4	1
K	4	10	2	0	12	2	2	2	6	3	0	5	0	6	0		4	28+	3-	3
F	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0		1	11+	3
R	1	1	3	0	1	0	1	2	1	0	0	1	0	0	1	7	0		3	2
Y	0	1	0	0	0	0	0	1	0	2	1	0	0	0	0	1	3	0		4
W	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	

(b)

	G	A	S	C	T	P	D	V	N	L	I	Q	M	E	H	K	F	R	Y	W
G		79+	65	4	45	25	34	16-	44+	9-	7-	19	6	23	8	31	4-	13	10-	3
A	23		110+	8	73+	35	33	63+	39	30-	24	39	11	46+	12	54	10-	21	14	4
S	14	35		5	128+	40	59+	31-	74+	17-	16	46	6-	32	16	53	8-	31	11-	4
C	0	0	0		7	4	0	6	0	3	3	1	0	2	0	0	2	0	0	1
T	7	21	43	0		31	29	48	47+	32	29	42	15	34	11	58+	15	29	10-	1
P	5	10	7	0	7		25	12-	16	7-	3-	11	1	22	8	22	3	9	4-	1
D	7	3	11	0	4	5		12-	61+	9-	9-	19	1-	47+	4	25	4-	9	2-	0
V	2	10	6	1	8	1	2		9-	65+	77+	13	19	18	7	21	19	16	12	0-
N	9	6	12	0	5	0	15	1		13-	8-	20	3	17	8	36	7-	10	9-	2
L	0	2	1	0	3	1	0	7	2		45+	21	30+	15	4	17	22	11	14	9
I	0	3	2	0	7	0	2	17	0	10		8	9	9	2-	7-	15	6-	6	5
Q	1	6	6	0	8	1	4	4	3	1	0		9	29+	10	38+	2-	18	5	2
M	0	0	1	0	4	0	0	2	1	7	3	1		3	3	6	5	2	2	1
E	4	6	6	0	8	5	12	3	2	1	2	9	0		4	31	3-	10	5-	0
H	3	2	0	0	0	2	0	0	1	0	0	0	0	1		8	5	3	8	2
K	5	11	5	0	14	2	2	2	7	2	0	8	0	6	1		4-	38+	3-	3
F	0	0	0	0	1	0	0	0	0	4	2	0	1	0	0	0		4-	23+	9+
R	2	3	4	0	5	1	2	2	2	1	0	4	0	0	2	9	0		4	2
Y	0	2	0	0	0	0	0	2	0	2	1	1	0	0	1	1	5	0		12+
W	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	

(c)